INDIRECT FLUOROMETRIC DETECTION OF ARSENIC AND SELENIUM OXYANIONS SEPARATED BY CAPILLARY ZONE ELECTROPHORESIS AND ION CHROMATOGRAPHY

By

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In order to satisfactorily assess water supplies for the presence of pollutants, it is necessary to be able to detect many different chemical species which are present at very low concentrations. The presence of arsenic and selenium is currently reported as total arsenic and total selenium, which does not give an accurate assessment of the water quality. It has been shown that the different species of these elements have different toxic effects on humans. The purpose of this research was to investigate the use of indirect fluorometric detection for the detection of arsenic and selenium oxyanions in water samples. Both capillary zone electrophoresis (CZE) and ion chromatography (IC) are utilized to separate these analytes, with a comparison of the results being presented.

Two different laser sources are evaluated with each separation technique. The first is a diode laser-based system. This excitation source was chosen because of its low

noise characteristics. The dyes which can be used at the wavelengths available with diode laser systems were found to react unfavorably with the capillary (in CZE), and the stationary phase (in IC).

The second system which was evaluated is a helium-cadmium (He-Cd) laser-based system. The selection of dyes which can be used with this source is much larger than with the diode laser excitation, and several different dyes are evaluated with each separation technique. Separation of four arsenic and selenium oxyanions is demonstrated with CZE and IC under the conditions necessary for indirect fluorometric detection. With a CZE separation, absolute detection limits in the femtomole range are achieved. This corresponds to concentration detection limits in sub-ppm range. Utilizing an IC separation, absolute detection limits are in the picomole range. This also corresponds to concentration detection limits in the sub-ppm range.

This research demonstrates the viability of using indirect fluorometric detection for the analysis of arsenate, arsenite, selenate and selenite in a simple water matrix with either CZE or IC separation. The detection limits achieved with these systems are comparable to those now published for these anions by other methods.

CHAPTER 1
INTRODUCTION

Environmental Significance

Our knowledge and understanding of the environment in which we live is increasing every day. One of the concerns addressed by the Environmental Protection Agency (EPA) is that of potable water quality. To be able to satisfactorily assess drinking water supplies for the presence of pollutants and their associated risks to humans and the environment, it is necessary to be able to detect the many different chemical species of an element which are present. This is due to the varying toxicities of the elements in their different oxidation states. The analytical chemist has been called upon to develop methods to meet these demands. Currently, the presence of arsenic and selenium in environmental samples is reported as total arsenic and total selenium. This does not give an adequate assessment of the drinking water quality. Recently, methods have been developed to separate and quantify the various species of arsenic and selenium present in water samples. These techniques include capillary zone electrophoresis (CZE) and ion chromatography (IC). Now that separation of these compounds is possible, improved detection methods are necessary to be able to detect ultra-trace levels of these compounds which may be present in water supplies. This would improve our ability to accurately assess water quality, and health hazards which may exist in the community.

Arsenic and Selenium

Arsenic has been recognized as a poison since antiquity. In high doses, it can cause nausea, vomiting, abdominal pain, brittle nails, dry flaky skin, hair loss, burning and tingling of extremities, mental confusion, and shuffling locomotion. In humans, it tends to concentrate in hair, nails and teeth, with only minor absorption into other body tissues. In spite of this fact, arsenic has been used for its "therapeutic" effects during the 1800s.[1] The most common formulation was named "Fowler's Solution" and contained 10 mg/ml of As_2O_3. It was prescribed for symptomatic relief of many conditions ranging from acute infections to epilepsy, asthma, psoriasis and eczema. For obvious reasons, this practice was abandoned with the advent of safer remedies for these conditions. Arsenic occurs naturally in nature, and is not often found as a free element but rather as part of a compound. In water, arsenic is most commonly found in the +5 oxidation state in the form of arsenic acid, H_3AsO_4, but can also be found in the +3 oxidation state as arsenous acid, $HAsO_2$. The acid dissociation constants for arsenic acid are $pK_{a1} = 2.20$, $pK_{a2} = 6.97$, and $pK_{a3} = 11.53$:[2]

$$\begin{aligned} H_3AsO_4 &\rightleftarrows H_2AsO_4^{-1} + H^+ \\ H_2AsO_4^{-1} &\rightleftarrows HAsO_4^{-2} + H^+ \\ HAsO_4^{-2} &\rightleftarrows AsO_4^{-3} + H^+ \end{aligned} \qquad (1\text{-}1)$$

The pK_a for arsenous acid is 9.18:[2]

$$HAsO_2 \rightleftarrows AsO_2^{-1} + H^+ \qquad (1\text{-}2)$$

Thus, in neutral water, As(V) would exist as a mixture of the singly protonated arsenate dianion and the diprotonated arsenate anion, where As(III) would exist mostly in its

nonionic protonated form. Arsenic also exists in methylated forms due to animal and plant metabolisms. The ratio of As(V):As(III) in a water system can vary greatly, for example, the ratio in seawater ranges from 0.1:1 to 10:1. Figures 1-1 and 1-2 show the effect of pH on the dissociation of H_3AsO_4 and $HAsO_2$, respectively.

Selenium is noted for its unusual trait of being a poison at high levels, yet being a necessary nutrient at lower levels.[3] It has been found that a concentration of up to 0.1 ppm is beneficial to humans, but a concentration of 0.4 ppm is considered toxic.[3] The presence of a small amount of selenium in the human body helps to prevent damage by oxygen to tissues, as does vitamin E.[3] Too little selenium is associated with a cardiovascular disease known as Keshan disease, and an osteoarthropathy disease named Kashin-Beck disease. The effects of ingesting too much selenium include an upset stomach, difficulty in breathing, and weakness. Over time, the symptoms include brittle nails, loss of hair and nails, skin lesions, tooth decay, and, in the later stages, convulsions, paralysis and motor disturbances.[3]

Selenium is most commonly found as sodium selenite, $NaSeO_3$, and sodium selenate, $NaSeO_4$. In water, selenium can exist in the Se(VI) oxidation state in the form of selenic acid, H_2SeO_4, which has $pK_{a1} = 0.3$, and $pK_{a2} = 1.92$:[2]

$$\begin{aligned} H_2SeO_4 &\rightleftarrows HSeO_4^{-1} + H^+ \\ HSeO_4^{-1} &\rightleftarrows SeO_4^{-2} + H^+ \end{aligned} \qquad (1\text{-}3)$$

Se(IV) can also occur in water in the form of selenous acid, H_2SeO_3, which has $pK_{a1} = 2.46$ and $pK_{a2} = 7.31$:[2]

Figure 1-1: Effect of pH on the dissociation of H_3AsO_4 in water: (■) H_3AsO_4; (▲) $H_2AsO_4^{-1}$; (□) $HAsO_4^{-2}$; (X) AsO_4^{-3}. Curve was constructed from pK values using a total H_3AsO_4 concentration of 1×10^{-4} M.

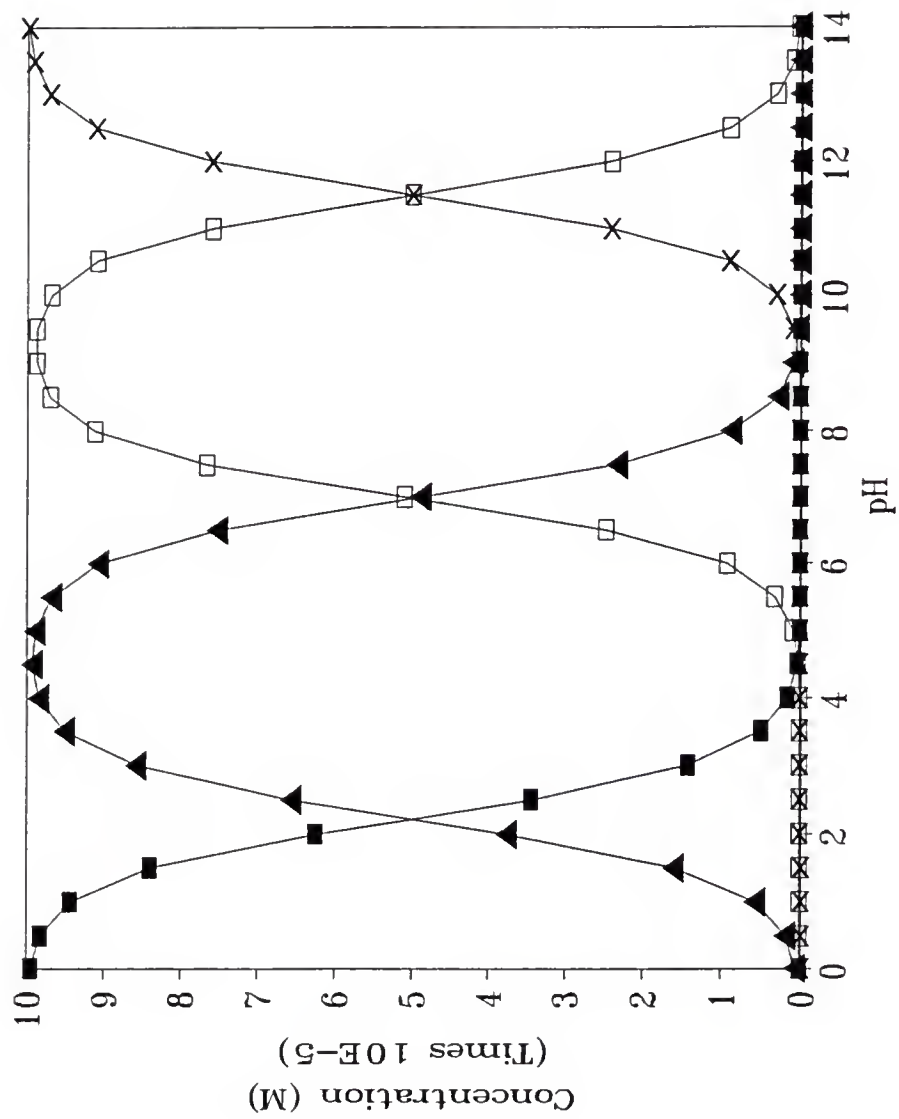

Concentration (M)
(Times 10E−5)
10
9
8
7
6
5
4
3
2
1
0
0
2
4
6
8
10
12
14
pH

Figure 1-2: Effect of pH on the dissociation of $HAsO_2$ in water: (■) AsO_2^{-1}. Curve was constructed from pK values using a total $HAsO_2$ concentration of 1×10^{-4} M.

Concentration of AsO2− (M)
(Times 10E−5)
10
9
8
7
6
5
4
3
2
1
0
0
2
4
6
8
10
12
14
pH

$$H_2SeO_3 \rightleftarrows HSeO_3^{-1} + H^+$$
$$HSeO_3^{-1} \rightleftarrows SeO_3^{-2} + H^+ \qquad (1\text{-}4)$$

In a typical water sample, Se(VI) is in the form of the selenate dianion, and Se(IV) exists as a mixture of deprotonated and singly protonated selenite anions. Selenium also exists as methylated forms produced by metabolic processes in the environment. Figures 1-3 and 1-4 show the effect of pH on the dissociation of H_2SeO_4 and H_2SeO_3, respectively.

The analysis of arsenic and selenium in environmental samples at the trace concentrations levels necessary for and accurate assessment of water quality has been a difficult problem. Various analytical techniques have been investigated including: flame atomic absorption spectroscopy,[4] graphite furnace atomic absorption spectroscopy,[5-6] theromochemical hydride generation-atomic absorption spectrometry,[7] inductively-coupled plasma-atomic emission spectroscopy,[8] suppressed ion chromatography with conductivity detection,[9] capillary zone electrophoresis with UV detection,[10] ion chromatography with atomic absorption spectrometric detection,[11] among others. Without some type of initial separation, most of these techniques can only determine total arsenic or total selenium present in a water sample.

EPA Regulations

In the early 1970s, it was indicated that a significant number of water supplies did not meet the established standards of water quality. It was at this point that legislation was established which provided the EPA with the ultimate authority over all water supplies. The Safe Drinking Water Act of 1974 (SWDA) established regulations for

Figure 1-3: Effect of pH on the dissociation of H_2SeO_4 in water: (■) H_2SeO_4; (▲) $HSeO_4^{-1}$; (□) SeO_4^{-2}. Curve was constructed from pK values using a total H_2SeO_4 concentration of 1×10^{-4} M.

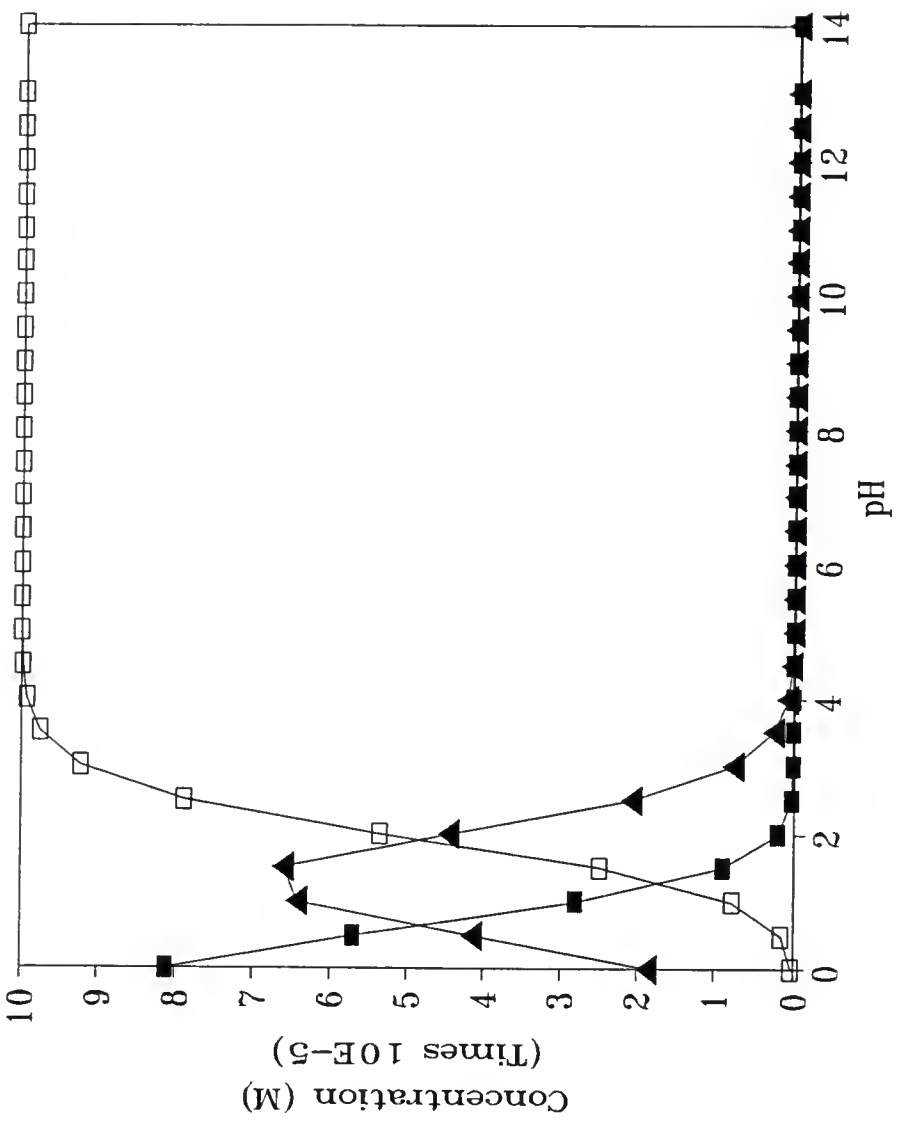
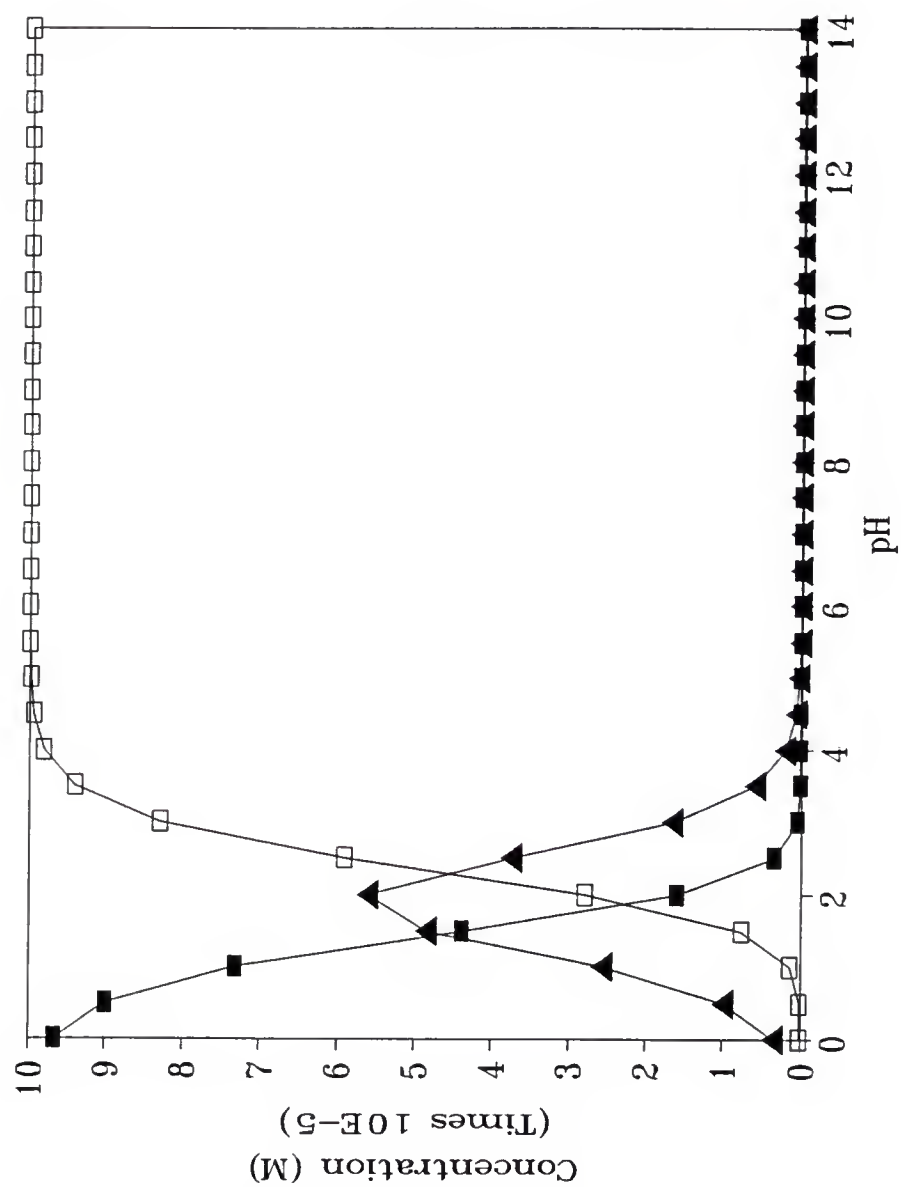

Concentration (M)
(Times 10E-5)
0
1
2
3
4
5
6
7
8
9
10
0
2
4
6
8
10
12
14
pH

Figure 1-4: Effect of pH on the dissociation of H_2SeO_3 in water: (■) H_2SeO_3; (▲) $HSeO_3^{-1}$; (□) SeO_4^{-2}. Curve was constructed from pK values using a total H_2SeO_3 concentration of 1×10^{-4} M.


Concentration (M)
(Times 10E-5)
10
9
8
7
6
5
4
3
2
1
0
0
2
4
6
8
10
12
14
pH

contaminants in drinking water supplies. To assure that the water we drink will cause no adverse effects on health, recommended maximum contaminant levels (RMCLs) were instituted. Since its beginnings in 1974, many amendments have been adopted to the SDWA. These new amendments have updated the SDWA with safer maximum contaminant levels (MCLs) as well as providing guidelines for the analysis of these contaminants. These guidelines, called "standard methods," allow all water samples across the country to be analyzed in a similar fashion, and then to be compared to one another, as well as with EPA standards.

Arsenic and selenium are both considered toxic metals, and appear on the list of contaminants which are regulated under the SDWA. The MCL in drinking water for arsenic has been set at 50 μg/L, and for selenium has been set at 10 μg/L. It should be noted that these levels are for the total arsenic and total selenium present in a water sample. As the toxicological studies continue, it is becoming evident that the different forms (and oxidation states) of these metals can cause different toxic effects. Because of this, it may become necessary to regulate each form separately, which necessitates the development of techniques to analyze these species in water samples. For routine analysis, techniques which are reliable, reproducible and relatively simple to perform are best. In addition, the instrumentation should be easy to maintain, and be rugged enough for daily use.

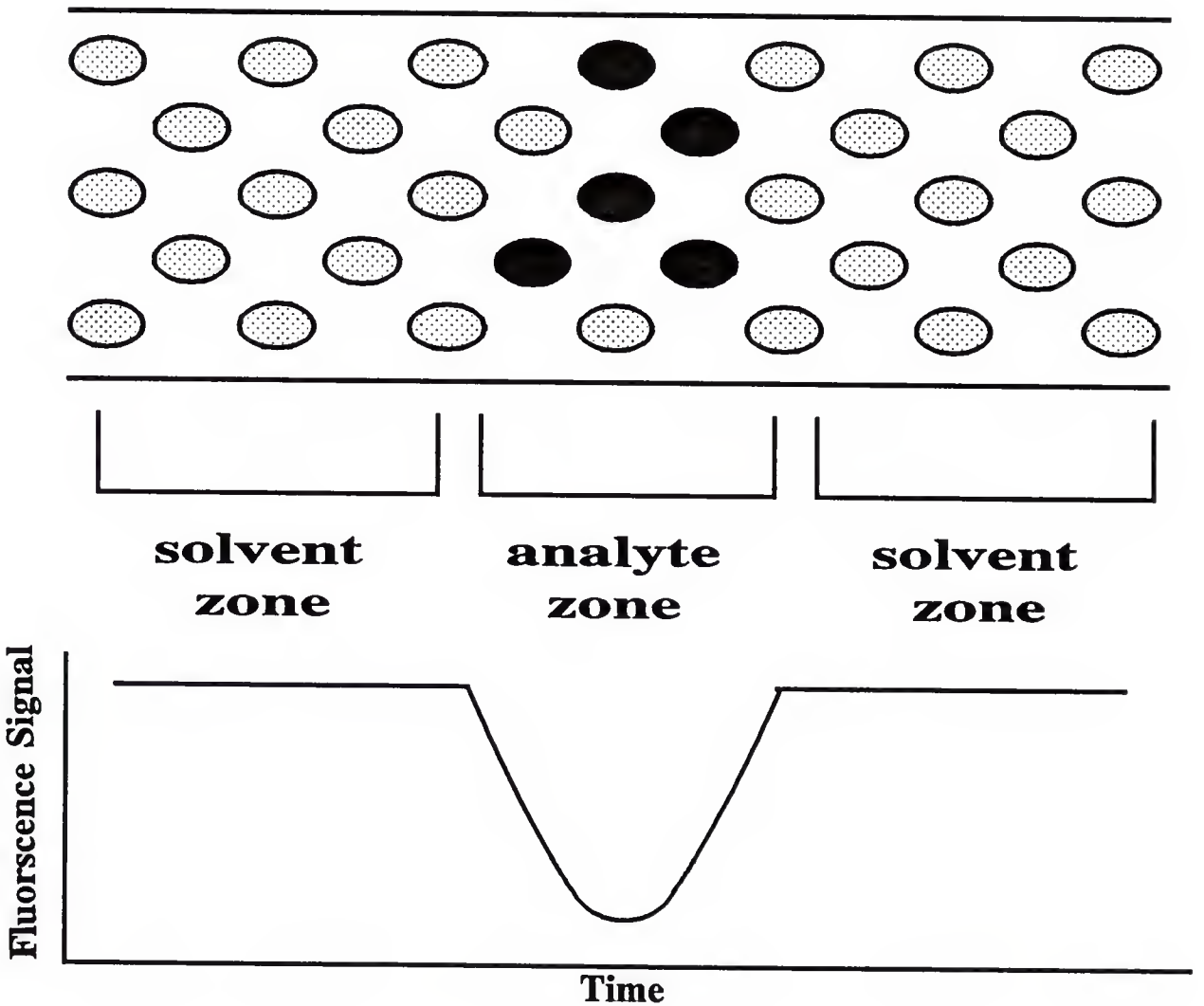
Indirect Fluorometric Detection

Small and Miller[12] were the first to characterize Indirect Photometric Detection (IPD) in 1982, although others had utilized indirect detection methods previously.[13-15] IPD was first developed for the universal detection of non-absorbing analytes separated by ion chromatography. This detection method eliminated the need for a suppressor column after the analytical column. The performance of Indirect Fluorometric Detection (IFD) is basically the same as for IPD except that it is more sensitive to the probe molecule, and therefore more sensitive than IPD. IPD and IFD have been applied to many separation techniques including ion chromatography,[12,16-22] HPLC,[23-24] thin-layer chromatography,[25] gas chromatography,[26] and capillary electrophoresis.[27-30] The use of indirect detection has been reviewed in several articles.[31-33]

Theory

Indirect fluorometric detection is based on a fluorescing probe species being present in the solvent, producing a constant background signal. When an analyte ion is present the probe is displaced and the fluorescence signal at the detector decreases. Since it is the presence of the analyte which causes the signal, and not the characteristics of the analyte, indirect detection is classified as a universal detection method. This allows the analysis of many analytes that cannot be detected by standard chromatography detectors, such as conductivity or UV-vis absorption. Figure 1-5 shows a schematic of an indirect detection measurement. The upper portion shows a segment of the capillary (or flow cell) which contains the probe species as part of the eluent. The analyte zone

Figure 1-5: Schematic of indirect fluorometric detection.


solvent zone
analyte zone
solvent zone
Fluorscence Signal
Time

in the figure shows how the probe is displaced by the analyte in that portion of the capillary. The lower portion illustrates a segment of the detector response. It shows how the fluorescence signal decreases when the analyte zone passes the detector window. For a chromatographic separation, the resulting chromatogram consists of a series of dips, rather than peaks.

Several parameters are extremely important when developing an indirect detection method. The first of these is the dynamic reserve (DR). This is related to the signal-to-noise ratio of the system, and provides a measure of the ability to detect a small change in a large fluorescence background signal.[31] For a laser-induced fluorescence background signal, the stability of the laser plays a major role in the value of DR. For a common laser-based signal, a typical DR is between 10^2 to 10^3. This DR is low when compared to some other indirect techniques: for indirect UV/vis absorption, DR is around 5×10^3; for refractive index detection, DR is around 10^6; and for polarimetry, DR is greater than 10^7.[34]

The second important parameter is the transfer ratio (TR). This is defined as the number of probe molecules displaced per analyte molecule.[31] Experimentally, it can be determined by measuring the signal produced by a known amount of probe species and dividing by the change in signal produced in the presence of a known amount of analyte:

$$TR = \frac{\text{slope of analyte calibration curve}}{\text{slope of probe calibration curve}}$$

The displacement which occurs can be a charge displacement, a volume displacement, a chromatographic partitioning, or a combination of all three. For ion chromatography

and capillary electrophoresis, a charge displacement mechanism prevails, based on the laws of eletroneutrality. Theoretically, this mechanism asserts that one analyte ion displaces one probe ion of the same charge, producing a TR of one.

Reversed-phase HPLC of neutral species relies on the solubility displacement mechanism for IFD. The presence of an analyte will cause the partitioning of the probe species between the stationary phase and the mobile phase to change. Usually analytes with shorter retention times than the probe species will induce a solubility enhancement of the probe into the mobile phase resulting in a positive peak. Those analytes with longer retention times than the probe species induce a solubility decrease, and negative peaks result.[35] The TR for HPLC is typically 10^{-4}.[36]

The detection limit for indirect detection is based on DR, TR and the concentration of the probe species, C_m. This relationship is expressed as:

$$C_{lim} = \frac{C_m}{(TR \times DR)} \qquad (1\text{-}6)$$

where C_{lim} is the theoretical concentration limit of detection.[31] According to this equation, the LOD can be decreased by lowering the concentration of the probe species while keeping the values of TR and DR constant. This is usually limited, however, by the flicker noise of the source. In most cases, when the concentration of the probe is lowered, the TR and DR values also change. In one laser-based approach, the fluctuations in the power of a He-Cd laser were compensated by special optics and a reference cell.[37] Another problem which can arise when conventional sources are used

is the fluorescence and/or absorption of an analyte at the detection parameters, which would interfere with the basic detection principle for indirect detection.

Probe Species

The selection of the probe species to be used in an analysis is of crucial importance. Many key factors must be considered. First, the probe must be able to provide a high background signal for the type of detection being utilized. For indirect fluorescence, the probe should have a high molar absorptivity and fluorescence quantum yield, which helps to achieve the maximum sensitivity for the system. Another consideration is the desired charge of the probe. The most efficient sensitivity occurs when the transfer ratio is one, which occurs with a charge displacement mechanism prevailing. This dictates that the probe species should have the same charge as the analyte of interest. This works especially well with ion chromatography and electrophoresis. A variety of probe species have been used with these separation techniques for both indirect UV-vis absorption and indirect fluorescence including: phthalate;[12,17,19,23] salicylate;[27,28,37-39] benzoate;[16] 2,4-dihydroxybenzoate;[22] quinine sulfate;[30] and methylene blue.[40] One other factor to be considered is the compatibility of the probe with the separation technique. It would be detrimental to use a probe which would inhibit the separation from occurring by irreversibly attaching to the stationary phase or the capillary wall. It is also necessary to determine if the probe is soluble in the chosen solvent, and if the probe interacts with the analytes in unfavorable ways.

Scope of Dissertation

The purpose of this dissertation is to investigate the use of indirect fluorometric detection for the detection of arsenic and selenium oxyanions in water samples. Both capillary zone electrophoresis and ion chromatography are used to separate these analytes, with a comparison of the results being presented. Two different laser systems are used with each separation technique. The first is a diode laser-based system. This type of laser system was chosen due to its low noise characteristics. Several near-IR dyes will be evaluated for their use as probe molecules in this type of system. The second is a He-Cd laser based system which utilized a commercial spectrofluorometer as the detection device. A variety of new probe molecules are evaluated with this system. This dissertation will show that this combination of CZE or IC with indirect fluorometric detection can provide a sensitive and universal alternative for the analysis of arsenic and selenium oxyanions in water samples.

CHAPTER 2
CAPILLARY ZONE ELECTROPHORESIS WITH INDIRECT FLUOROMETRIC DETECTION

Theoretical Aspects of Capillary Electrophoresis

Capillary zone electrophoresis (CZE) is a method which utilizes an electric field to facilitate a separation of charged species. The advantages of CZE over the more common technique of high performance liquid chromatography (HPLC) include the smaller sample sizes needed, and the higher number of theoretical plates achievable. Since it is a similar technique to HPLC, many of the terms used in CZE have been adopted from HPLC. Mikkers, Everaerts and Verheggen[41] were the first to perform CZE in narrow bore tubes. They were able to achieve separation of 16 anions in less than 10 minutes. Jorgenson and Lukacs[42] were the next to describe a system using open-tubular glass capillaries for performing zone electrophoresis. CZE has been used for the analysis of anions and cations spanning from small inorganic ions to large biomolecules. The separation of neutral molecules has also been achieved by using micelles in the buffer solution.[43-44] The neutrals partition between the micelles and the buffer solution, which drives the separation. This type of analysis has been very effective for this type of separation.

Definitions

A basic CZE system consists of a fused silica capillary tube filled with a buffer solution, and with each end of the capillary immersed in a vial also containing the buffer solution. An electrode is placed in each vial, and a potential is applied across the capillary. Detection is normally performed "on-line" by removing a small section of the polyimide coating which coats the capillary. This window in the capillary coating then becomes the detector "flow cell". When the potential is applied, each ion will then move toward one of the electrodes, and pass the detector window, according to its effective charge and size, hence its mobility. The velocity at which each ion moves is called the migration velocity and can be described by the following equation:

$$\nu = \frac{\mu V}{L} \tag{2-1}$$

where ν is the migration velocity (m/s), μ is the mobility (m^2/Vs), V is the total applied voltage (V), and L is the total length of the capillary (m).[45] The time required, t_L, for the analyte zone to migrate the entire length of the tube is:

$$t_L = \frac{L}{\nu} = \frac{L^2}{\mu V} \tag{2-2}$$

Zone broadening is caused by molecular diffusion, and the spatial variance, σ_L^2, is given by the Einstein equation:

$$\sigma_L^2 = 2Dt \tag{2-3}$$

where D is the molecular diffusion coefficient of the solute in the zone (m^2/s). The efficiency of the CZE separation can be expressed in terms of theoretical plates, as is done in chromatography. The number of theoretical plates, N, can be defined as:

$$N = \frac{L^2}{\sigma_L^2} = \frac{\mu V}{2D} \qquad (2\text{-}4)$$

and can be calculated from peak profiles as in chromatography by using the formula:

$$N = 5.54 \left(\frac{t_L}{w_h} \right)^2 \qquad (2\text{-}5)$$

where w_h is the width of the peak at half of its height (s), and t_L is the migration time (s). The equation for the height of a theoretical plate (H) is then given as:[45]

$$H = \frac{L}{N} \qquad (2\text{-}6)$$

In CZE separations, hundreds of thousands of theoretical plates can be achieved. This provides an enhanced separating ability to that of conventional HPLC.

In CZE, there is also another force which is affecting the separation. This is the electroosmotic mobility, which is due to the induced bulk flow of the liquid buffer. The inner wall of the fused silica capillary possesses a negative charge due to the silanol groups of the silica. The positive ions in the buffer migrate toward these silanol groups. When the electric field is applied, these positively charged ions move toward the negative electrode, thus creating the electroosmotic flow. The apparent mobility of an ion, μ_{app}, is the critical parameter in CZE; μ_{app} is the combination of the electroosmotic mobility of the buffer ,μ_{eo}, and the electrophoretic mobility of that ion ,μ_{ep}. This relationship is given by:

$$\mu_{app} = \mu_{ep} \pm \mu_{eo} \qquad (2\text{-}7)$$

In the case where the electroosmotic mobility and the electrophoretic mobility of the

analyte are in the same direction, the values are added. If they are moving in opposite directions, the values are subtracted. Many times the detection is performed on-line by making a detector window at some designated length of the capillary. The apparent mobility can be obtained in terms of easily measurable quantities and thus calculated by the following equation:

$$\mu_{app} = \frac{L_D L_T}{t_{app} V} \tag{2-8}$$

where L_D is the length of the capillary from the injection end to the detector window, and L_T is the total length of the capillary. The electroosmotic mobility of the buffer can be calculated in a similar fashion by injecting a neutral molecule, one that will only move through the capillary due to the force of the flowing buffer, and recording its migration time (t_{mg}):

$$\mu_{eo} = \frac{L_D L_T}{t_{mg} V} \tag{2-9}$$

By combining equations (2-6) and (2-7) it can be shown that:

$$\mu_{ep} = \frac{L_D L_T}{V} \left[\frac{1}{t_{mg}} + \frac{1}{t_{app}} \right] \tag{2-10}$$

It is important to report data in terms of electrophoretic mobility, instead of migration time. There are several reasons for this: first, it is sometimes hard to control the electroosmotic flow, making mobility values more reproducible than migration times; second, migration times are dependent on many experimental conditions, which can vary from day-to-day and run-to-run; third, by reporting results as electrophoretic mobilities,

direct comparisons can be made easily and effectively; and lastly, it allows you to correlate the electrophoretic behavior of a solute with its structure.

The resolution (R) of two peaks can also be calculated in a similar fashion to that which is used in chromatography:

$$R = \frac{1}{4} \frac{\Delta\mu_{app}}{\bar{\mu}_{app}} \sqrt{N} \qquad (2\text{-}11)$$

where $\Delta\mu_{app}$ is the difference of the two mobilities, $\bar{\mu}_{app}$ is the average of the two mobilities, and N is the number of theoretical plates.

There are basically two types of injections which can be used in CZE. The first of these is hydrodynamic injections. For this type of injection, one end of the capillary is removed from the buffer vial and placed in a vial containing the sample. This vial is then raised a predetermined height for a specified time. During this time, the sample is drawn up into the capillary by siphoning action. The sample vial is then lowered, and the end of the capillary is returned to the buffer vial. The separation is then performed. The volume injected, V_{inj} (ml), can be calculated by the Poiseuille equation:

$$V_{inj} = \frac{\rho g \Delta h \pi D^4 t_{inj}}{128 \eta L_T} \qquad (2\text{-}12)$$

where ρ is the density (g/cm^3), g is the gravitational constant (cm/s^2), Δh is the height difference (cm), D is the inner diameter of the capillary (cm), t_{inj} is the injection time (s), η is the viscosity of the buffer solution (g/cm s), and L is the length of the capillary (cm). Hydrodynamic injection provides the most precise injection of a sample because it is based strictly on volume loading of the sample.

The second type of injection is called electrokinetic injection. With this technique one end of the capillary, along with the corresponding electrode, are placed in the sample vial. A voltage is then applied for a designated amount of time. During this time, the electroosmotic flow and the individual electrophoretic mobilities determine the amount of sample which is introduced into the capillary. After the allotted time, the end of the capillary and the electrode are returned to the buffer vial, and the separation is performed. The amount of analyte injected can be calculated by the equation:

$$Q_{inj} = \frac{\pi r^2 c_s (\mu_{ep} + \mu_{eo}) E t_{inj} \lambda_b}{\lambda_s} \qquad (2\text{-}13)$$

where Q_{inj} is the amount of sample injected (mol), r is the radius of the capillary (m), c_s is the concentration of the sample (mol/L), μ_{ep} is the electrophoretic mobility of the sample ($cm^2/V\ s$), μ_{eo} is the electroosmotic mobility ($cm^2/V\ s$), E is the electric field strength (V/cm), t_{inj} is the injection time (s), λ_b is the conductivity of the sample buffer electrolyte, and λ_s is the conductivity of the sample. With electrokinetic injection, ions with higher mobilities are concentrated in the injection volume, whereas ions with lower mobilities are diluted in the injection volume. This characteristic can be useful when a preconcentration step is desired, but can be detrimental to an analysis if this is not considered.

Types of Analytes

As was mentioned earlier, a wide variety of analytes can be separated and detected using CZE systems. These include inorganic anions,[46-49] inorganic cations,[50]

organic acids,[51-53] organic bases,[54] amino acids,[42,55-58] peptides,[59] and proteins.[59] Since these analytes possess many different characteristics, in order to separate them all, some basic experimental parameters need to be altered. In the conventional configuration, the electroosmotic flow is toward the negative electrode with the sample being injected in the end of the capillary near the positive electrode. With this setup, the cations will be detected first (since they are moving with the electroosmotic flow toward the negative electrode), the neutrals will be carried with the flow, and the anions (which are moving against the flow) will be detected last, if at all.

Anions. In some cases, where the electroosmotic flow is not very fast, and the analytes are small inorganic anions, they will stay at the injection end and not be detected. One way to overcome this is to reverse the voltage. Since the injection end would now be negative, the anions would migrate through the capillary, and pass the detector window. There could still be a problem, however, since the anions continue to move against the electroosmotic flow (which is moving toward the negative electrode). In this case, a slow electroosmotic flow would be desired, so as not to "push" the analytes back toward the negative electrode. This can be accomplished by treatment of the capillary with a chemical derivitizing agent, such as trimethylchlorosilane. This process, called silylation, changes the charge density of the capillary wall.[28] The next option is to reverse the electroosmotic flow. This can be accomplished by coating the inner surface of the capillary which will change it to carry a positive charge. In this configuration, the anions in the buffer will migrate toward the walls, and upon the application of an electric field, the solution will flow toward the positive electrode.

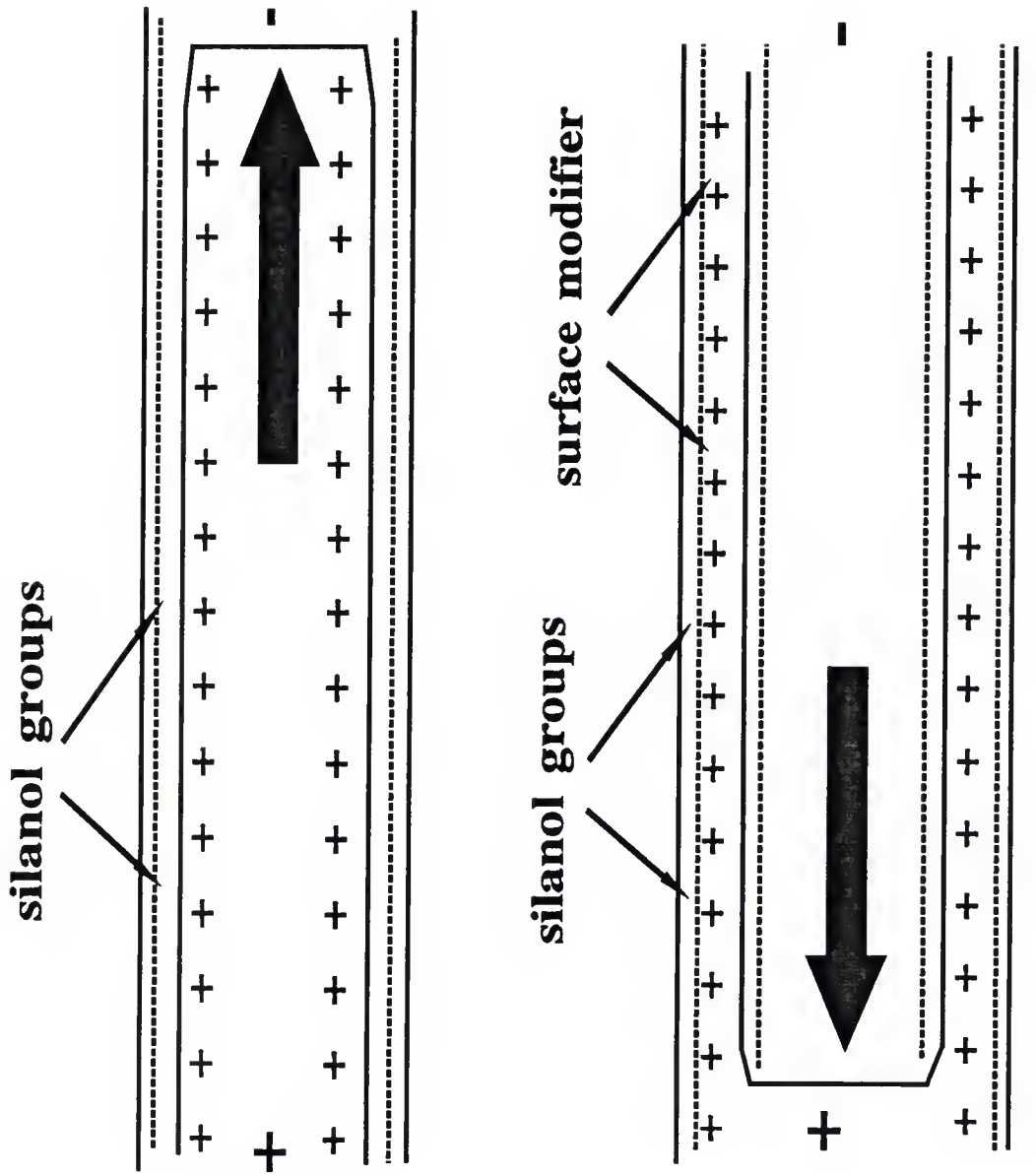
Figure 2-1 shows a schematic of this flow reversal process. This reversal has been accomplished by adsorbing a coating onto the capillary wall. The most common type of coatings involve cationic surfactants which are added to the buffer system. Some of the surfactants which have been shown to reverse the electroosmotic flow include: dodecyltrimethylammonium bromide (DTAB),[60] tetradecyltrimethylammonium bromide (TTAB),[60] hexadecyltrimethylammonium bromide (CTAB),[60] and cetyltrimethylammonium chloride.[61] Waters Chromatography Division has even developed their own "electroosmotic flow modifier" to be used with their CZE system for the analysis of anions by indirect UV-absorption spectroscopy.[48-49] Their modifier is a proprietary compound, and all they will reveal is that it is a type of alkylammonium compound. Another type of compound which has been used to reverse the electroosmotic flow is diethylenetriamine (DETA) by Dionex Corporation.[62]

Detection Methods

Due to its enhanced separating abilities, CZE is becoming a more popular technique for analysis of ions. Analytical chemists are constantly trying to achieve lower detection limits for more and more compounds. Many advances in detection methods have been explored in conjunction with CZE. Initially, UV-vis absorption and conductivity were the methods of choice.

Absorption. Absorption detection is easily adaptable to CZE, since a wide variety of analytes do absorb in this region, and the detection can be performed on-line.[54,63-64] The drawback to this method is its decreased sensitivity from the use of small inner

Figure 2-1: Schematic of electroosmotic flow reversal: (a) conventional flow (toward the negative electrode); (b) reversed flow (toward the positive electrode).


silanol groups
surface modifier
silanol groups

diameter capillaries, giving a small pathlength (normally < 100 μm). One novel approach which was investigated to provide increased sensitivity is called axial-beam absorbance detection.[65] In this technique, the light beam is directed along the capillary axis. The resulting pathlength is then equal to the analyte band width. A 60-fold improvement in path length was achieved.

Conductivity. The use of conductivity detection can provide an increase in sensitivity, but it also requires some type of sample flow cell other than the capillary itself. Several designs have been published in which a capillary detector cell was designed. The detector cell had a similar inner diameter as the capillary, and was adapted to allow platinum electrodes to be embedded into the wall.[51,64] An on-column conductivity detector was designed by Huang and co-workers consisted of two platinum wires sealed into 40-μm holes penetrating the walls of the fused-silica capillary.[66] The disadvantages of this technique are mainly baseline drift, and poor detection limits.

Fluorescence. Fluorescence detection has also been utilized with CZE. As with absorbance detection, on-line detection is easily achieved. The use of fluorescence provides an enhancement of sensitivity over absorption, but the range of analytes which can be detected decreases. Initially, a broad source was used for excitation (for example, a xenon-arc lamp),[67] but more recent advances involve the use of lasers as excitation sources. Currently, the most sensitive detection limits, in the attomole range, have been achieved with laser-induced fluorescence (LIF).[58,68-69] Lasers which have been used as excitation sources include the cw helium-cadmium (He-Cd),[70] the cw argon ion (Ar^+),[71] and a cw semiconductor (diode) laser[40]. To extend the applicability of fluorescence

detection to non-fluorescence molecules, fluorescent labeling is often used. This type of analysis has been performed with amino acids, with zeptomole detection limits for fluorescein thiohydantoin derivatives of amino acids being reported by Wu and Dovichi.[58]

Mass spectrometry. Mass spectrometry is potentially an ideal method for CZE. It would allow direct identification of each analyte zone, as well as low detection limits. Electrospray ionization was the first type of interface to be used.[72] The difference between MS detection and the previously mentioned ones is that the detection end of the capillary is not placed into a vial, but rather into the MS interface. A metal needle or a thin film of metal deposited on the capillary end ensures electrical contact and allows the appropriate voltage potential to be created along the capillary. One problem which arises with MS detection occurs with aqueous buffers. Many times organic modifiers are added to the buffer system to increase its volatility. Other researchers have developed a fast-atom bombardment-mass spectrometry (FAB-MS) interface for a CZE system.[73-74] Detection limits in the femtomole range have been reported with this type of system.

Indirect detection methods. Both indirect absorption and indirect fluorescence have been used for detection with CZE. Kuhr and Yeung were the first to utilize and indirect fluorescence detection scheme for the analysis of amino acids separated by CZE.[27] Since that time, several studies have been performed with indirect absorption detection for such analytes as: organic acids,[52-53] amino acids,[52] inorganic anions,[48-49,75-76] polyamines,[56] and inorganic cations.[77] Jones and Jandik have been successful in separating 30 anions in 89 seconds using CZE with an electroosmotic flow modifier and indirect absorption detection.[48] Indirect fluorescence has also been investigated further.

Analytes detected by this method include: nucleotides,[28] nucleosides,[28] sugars,[29] inorganic cations,[30,78] amines,[30] amino acids,[40] and inorganic anions.[47,78] Several review articles covering indirect detection have also been published.[79-80] Indirect detection has the advantage of being a sensitive and universal detector. It eliminates the need of pre- or post-column derivitization, and offers the possibility of one calibration curve for a series of analytes.

Using a Diode Laser as an Excitation Source

One way to reduce the detection limits in an indirect fluorometric detection system is to increase the signal-to-noise ratio. This can be achieved by increasing the intensity or decreasing the noise of the excitation source, which is most often a laser. Diode lasers (also called semiconductor lasers) can offer this improved stability. For example, the power stability of a He-Cd laser is about 0.1%, whereas the power stability of a diode laser is around 0.004%. This characteristic alone makes diode lasers an interesting option as an excitation source for indirect fluorometric detection.

Introduction to Diode Lasers

The first diode laser was demonstrated in 1962,[81] but continuous wave (cw) operation at room temperature was not reported until 8 years later.[82] Diode lasers offer several advantages over conventional lasers. They are compact, efficient and cheap. A diode laser, complete with heat sink, power stabilizer and protective window can be as small as 50 mm^2, yet deliver up to 5000 mW of power. The efficiency of the diode laser

is typically 30%, as compared to 2% for the He-Ne laser. The cost of a diode laser system can range from $100 to $10,000, depending on the stability, power and wavelength desired. The lifetime of the diode laser is given at 10^6 hours. This is in contrast to a He-Cd laser, whose plasma tube lifetime is approximately 4000 hours, and costs over half of the original price of the system to replace. Diode lasers are also extremely rugged. Since there are no plasma tubes or other fragile optical components, the diode laser would be an ideal candidate for field instrumentation.

Diode lasers are solid state electronic emitters of electromagnetic radiation.[83] The are similar to light-emitting diodes (LEDs) except that the diode laser possesses a cavity to induce lasing. They are commonly made out of gallium doped arsenide, which has an energy gap that corresponds to wavelengths in the red to near-IR region of the spectrum. The one major drawback of the diode laser is the wavelength availability. Currently, they are only available at a few wavelengths between 670 nm and 3000 nm. They are tunable (over a 20-30 nm range) but normally it is not possible to have continuous tunability over this wavelength range, because of the tendency of diode lasers to mode hop, or instantaneously jump from one wavelength to another.

One major drawback of the diode laser is the wavelength availability. Working in the red and near-IR region of the spectrum decreases the background fluorescence due to contaminants and impurities in a sample; however, it also limits the number of compounds which can be detected by LIF. Recently, there has been research involving the labelling of compounds with red and near-IR absorbing fluorophores. Several different groups of chemicals possess the qualities necessary for this type of analysis.

The first group consists of thiazines and oxazines. These compounds contain amine groups which are useful for labelling purposes. Representatives of this group include Nile Blue, Methylene Blue and Oxazine 750. Another group consists of a class of compounds called phycobiliproteins. They show high molar absorptivities and good quantum yields, however, they are very large molecules, and therefore their applications are limited. A third group of compounds consists of the cyanine dyes. These compounds have high molar absorptivities and are strongly fluorescent, and for these reasons they are commonly used as laser dyes and as fluorescent bioprobes.[84] A disadvantage to the cyanine dyes is that their fluorescence and solubility is rapidly decreased in aqueous solvents.[85] Nevertheless, cyanine dyes have been used as fluorescent labels in several studies.[86-90] Table 2-1 gives a list of some of the above mentioned dyes, along with some of their characteristics.

Experimental Section

Electrophoresis system. A schematic of the instrumental setup is shown in Figure 2-2. The CZE system was built in the laboratory. It consists of a Bertan Model 205B high voltage power supply (Hicksville,NY 11801), with a range of -50 to +50 kV. A plexiglass box, with a safety interlock, was designed to house the vials of buffer in which the capillary ends are immersed. Platinum wire was used for the electrodes, which are also immersed in the buffer solutions. The fused silica capillary tubing was obtained through Polymicro Technologies (Phoenix,AZ 85017). Due to the configurations of the entire system, a capillary length of 100 cm was used throughout. Two different capillary

Table 2-1: Fluorescent near-IR dyes for possible use with diode laser systems.

Name	Molecular Formula	CAS #	λ_{ex} (nm)	λ_{em} (nm)	ϵ_{max} $M^{-1}cm^{-1}$	Y_F
Rhodamine	$C_{26}H_{26}ClN_3O$	[101027-54-7]	682[a]	700[a]	89,500[a]	0.39[a]
Methylene blue	$C_{16}H_{18}ClN_3S$	[61-73-4]	668[a]	683[a]	66,600[a]	---
Nile blue	$C_{20}H_{20}ClN_3O$	[56996-76-0]	640[a]	672[a]	77,500[a]	---
Oxazine 750	$C_{24}H_{24}ClN_3O$	[85256-40-2]	673[a]	691[a]	82,500[a]	---
IR-125	$C_{43}H_{47}N_2S_2$	[3599-32-4]	780	815	180,000	0.13[c]
IR-132	$C_{52}H_{48}N_3O_4S_2$	[62669-62-9]	764	824	210,000	---
IR-140	$C_{38}H_{34}Cl_2N_3O_4S_2$	[53655-17-7]	803	821	180,000	1.0[d]
IR-144	$C_{58}H_{72}N_5O_4S_2$	[54849-69-3]	698[b]	708[b]	127,000	---
DTTC	$C_{25}H_{25}N_2S_2$	[3071-70-3]	746[b]	777[b]	---	0.38[c]
DTDC	$C_{23}H_{23}N_2S_2$	[514-73-8]	647[b]	668[b]	---	0.73[c]
DOTC	$C_{25}H_{25}N_2O_2$	[15175-43-0]	678[b]	703[b]	---	0.63[c]
HITC	$C_{28}H_{33}N_2$	[121518-87-4]	736[b]	764[b]	240,000	0.28[c]
HDITC	$C_{36}H_{37}N_2$	[95235-08-8]	771[b]	805[b]	---	---
DDTC	$C_{32}H_{29}N_2$	[100835-15-2]	765[c]	855[c]	---	0.16[c]
DQDC	$C_{27}H_{27}N_2$	[14187-31-6]	765[c]	835[c]	---	0.001[c]
DQTC	$C_{29}H_{29}N_2$	[17695-32-8]	825[c]	865[c]	---	0.035[c]

[a] Values in water from reference 89.
[b] Values in methanol from reference 85.
[c] Values in dimethylsulfoxide from reference 91.
[d] Values in methanol from reference 92.
[e] Values in methanol from reference 93.

Figure 2-2: Illustration of diode laser based CZE system. Inset contains schematic of instrumentation.

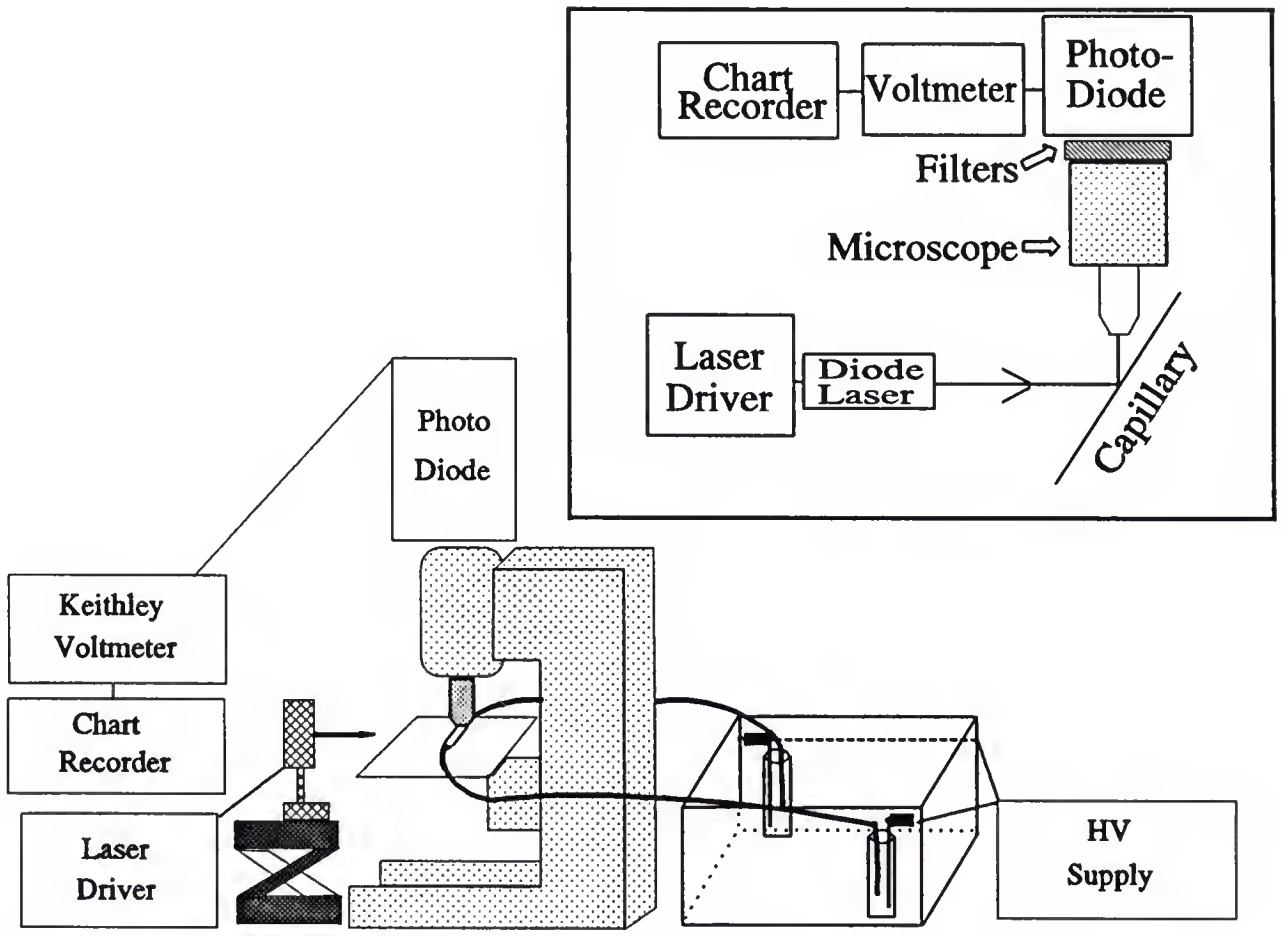


Chart Recorder
Voltmeter
Photo-Diode
Filters
Microscope
Laser Driver
Diode Laser
Capillary
Photo Diode
Keithley Voltmeter
Chart Recorder
Laser Driver
HV Supply

inner diameters, 50 μm and 75 μm, were investigated during the experimentation. A manual injection mode was utilized throughout. This was accomplished by placing the injection end of the capillary into a vial containing the sample solution, raising it up to a predetermined distance (normally around 10 cm), for a set amount of time (ranging from 60 s to 5 s).

Excitation system. The excitation was accomplished by the use of diode lasers. The first diode laser used was a Spectra Diode Labs SDL-2422-H1 diode laser controlled by a Spectra Diodes SDL 800 laser driver with temperature control (San Jose,CA 95134). This laser emitted at 796 nm, with an output power of 200 mW being utilized. Problems arose with its stability, so a second laser was tried. This laser was a Mitsubishi 4402 diode laser (Tokyo,Japan) driven by the Spectra Diodes SDL 800 laser driver, with an ILX LDT-5910 thermoelectric temperature controller. This laser emitted at 780 nm and had a power output of 3 mW at an operating current of 49 mA. The laser beam was focused onto the section of capillary which was being used at the detector window. The beam diameter was approximately 150 μm.

Detection system. On-line detection was performed by removing a small portion of the polyimide coating from the fused silica capillary. Two different techniques were evaluated for the removal of this coating. The first, and easiest, was to burn the coating off by heating a portion of the capillary with a match (or lighter). The burnt coating was then removed by gently cleaning the capillary with methanol. While this is an effective method, care must be taken, since the heating processes leaves the uncoated capillary weak and brittle. The second method is to dip the portion of the capillary into hot

sulfuric acid. This too is an effective method, and while it does not cause the capillary to become weak and brittle, the need to heat concentrated acid is a cause of concern. The portion of the capillary which serves as the detector window was held on a microscope stage by a specially modified holder. The fluorescence was collected at 90° by a Nikon 20x microscope objective. Cutoff filters were placed before the detector to reduce the laser scatter from the capillary. Several filter combinations were evaluated, with the best combination being a Corion RG 850 high-pass filter (Holloston,MA 01746) and a Kodak #87C Wratten filter (Rochester,NY 14650). The detector was a Hamamatsu HC210-3314 red-sensitive photodiode with amplifier (Bridgewater,NJ 08807). The output was directed to a Keithley 182 Sensitive Voltmeter (Cleveland,OH 44169), followed by a Fisher Series 500 chart recorder (Pittsburg,PA 15219). The voltmeter settings included a 16.6 ms integration time, and the digital filtering on medium response. The output offset was used to compensate for the large background fluorescence signal, and to allow for close analysis of the background noise and the analyte signals. The entire microscope, including the detector, was contained in a black box. This helped to reduce scatter and external sources of light from reaching the detector.

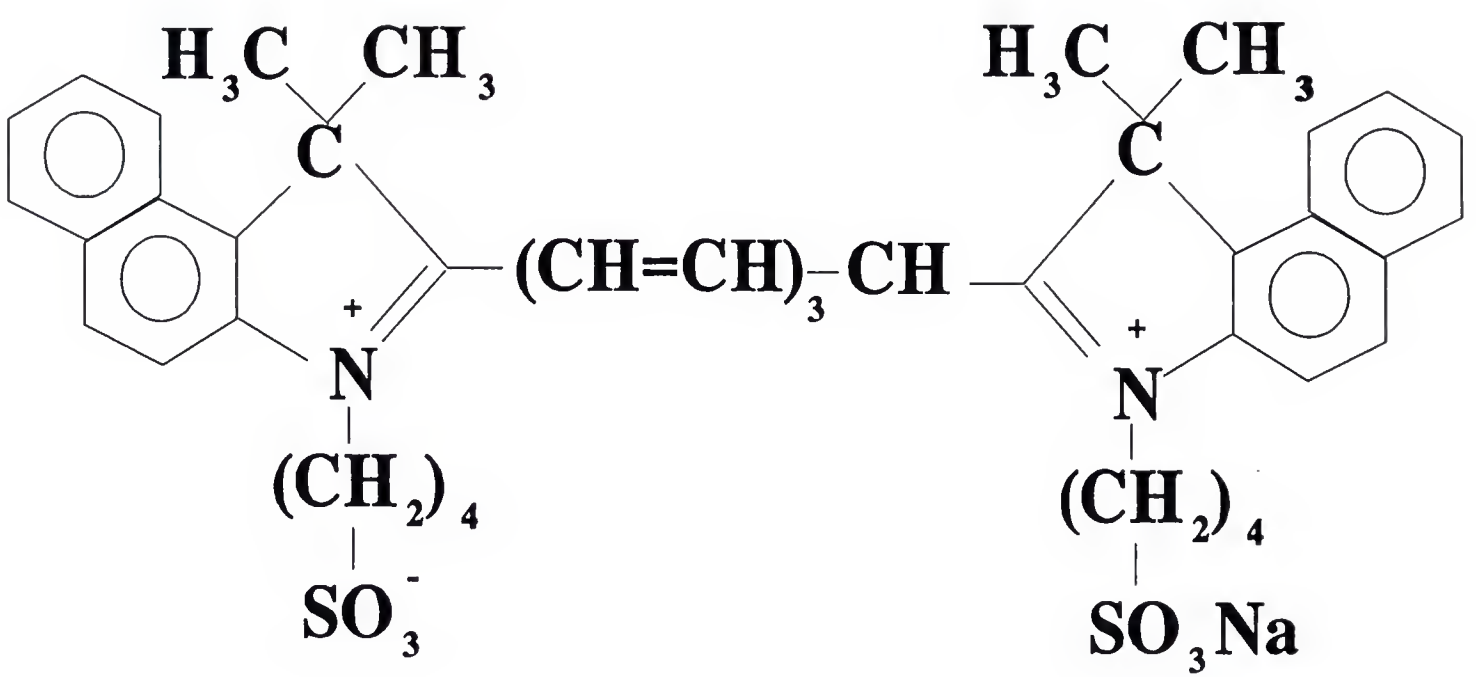
Chemicals. The analyte solutions were made from their sodium salts. Sodium arsenite, sodium arsenate, sodium selenite, and sodium selenate were obtained from Sigma Corporation (St.Louis,MO 63178). The near-IR dye, IR-125, was obtained from Exciton Corporation (Dayton,OH 45431). The cationic surfactants, dodecyltrimethylammonium bromide (DTAB), tetradecyltrimethylammonium bromide

(TTAB), and hexadecyltrimethylammonium bromide (CTAB), used as flow modifiers were also obtained from Sigma Corporation. The phosphate buffer was prepared with sodium phosphate monobasic and sodium phosphate dibasic from Fisher Scientific (Pittsburg,PA 15219). The pH was adjusted, when necessary, with a dilute solution of sodium hydroxide. Barnstead nanopure water (Dubque,IA 52004) was used to prepare all of the solutions. Methanol and acetonitrile were HPLC grade and also obtained through Fisher Scientific.

Optimization of Parameters

Probe species. The probe species which was chosen for these experiments was IR-125. Its formal chemical name is anhydro-1,1-dimethyl-2-[7-[1,1-dimethyl-3-(a4-sulfobutyl-2-(1H)-benz(e)indolinylidene]-1,3,5-heptatrienyl]-3-(4-sulfobutyl)-1H-benz(e)indolinium hydroxide sodium salt; its Chemical Abstracts Services (CAS) number is [3599-32-4]. It is commonly known as indocyanine green, or ICG, which is associated with its use as an indicator. The structure of IR-125 is given in Figure 2-3. Its λ_{ex} is at 784 nm, with its λ_{em} at 805 nm. IR-125 was chosen for several reasons. First, IR-125 is a zwitterionic salt, which has a slight negative charge at a pH above 3.27 (its pK_a in aqueous solutions). Common uses of IR-125 include its use as a laser dye, and as a clinical indicator dye for testing *in vivo* blood flows and hepatic functions in animals and humans.[94] As was mentioned earlier, a displacement based on a charge mechanism is desired, and since the analytes of interest are anionic in nature, it is necessary for the probe species to also be anionic. Another reason for choosing IR-125 is its solubility

Figure 2-3: Structure of probe species, IR-125.


H3C CH3
C
(CH=CH)3 CH
+
N
(CH2)4
SO3-
H3C CH3
C
+
N
(CH2)4
SO3Na

characteristics. It is one of the few near-IR dyes which is soluble in aqueous solvents. Although it is soluble in water, it is not highly fluorescent in this type of solvent. Figure 2-4 shows the results of a study to determine the effects of increasing organic solvent on the fluorescence signal of IR-125. This study was performed on a SPEX Fluorolog spectrophotometer system, which utilized an R928 PMT as the detector. It shows that there is a linear relationship between the percentage of organic solvent present, and the fluorescence intensity of IR-125. Since organic solvents can be used in electrophoresis to modify the electroosmotic flow, this could be worked into the electrophoretic separation parameters.

The first objective was to determine if the CZE system was operating properly, and to assure that the position of the capillary, an associated optics, were aligned. Using a buffer solution containing 30% methanol and 1 mM phosphate buffer, injections of IR-125 were performed. The background signal of the buffer solution was 12.8 mV with a N_{rms} of 0.02 mV, giving a S/N ratio of 642. With this system, a LOD of 4×10^{-18} moles (4 attomoles) was achieved for IR-125. The reproducibility of the migration time (μ_{app}) was less than 3%.

The next step was to evaluate the behavior of the IR-125 as a component in the buffer system. Solutions containing varying amounts of IR-125, methanol, and phosphate buffer were prepared and evaluated in the CZE system. Unfortunately, many reproducibility problems arose. Inexplicable oscillations in the background fluorescence signals would occur. Substantial drops in the background would also be observed. An electroosmotic flow was created, and when water was injected at the positive electrode,

Figure 2-4: Effect of organic modifiers on the fluorescence signal of IR-125;
(a) □ represents data with increasing amounts of acetonitrile;
(b) ⧗ represents data with increasing amounts of methanol.

Intensity (for Acetonitrile) (cps)
(Thousands)
Intensity (for Methanol) (cps)
% Solvent
0
10
20
30
40
50
60
70
80
90
100
500
1000
1500
2000
2500
3000
3500
4000

an electroosmotic flow marker was observed, as was a dip in the baseline due at the μ_{app} of IR-125. For a buffer solution consisting of 1.29 x 10^{-6} M of IR-125, 10% methanol and 1 mM phosphate buffer, the electroosmotic mobility (μ_{eo}) was calculated to be 4.6 x 10^{-4} cm^2/Vs, and the mobility of IR-125 ($\mu_{IR\text{-}125}$) was calculated to be -1.2 x 10^{-4} cm^2/Vs. For a buffer solution made of 1.29 x 10^{-6} M IR-125, 30% methanol and 1mM phosphate buffer (pH 6.6), the μ_{eo} was calculated to be 2.83 x 10^{-4} cm^2/Vs, and the $\mu_{IR\text{-}125}$ was calculated to be -1.0 x 10^{-4} cm^2/Vs. The decrease in the electroosmotic mobility was due to the higher percentage of methanol present in the buffer solution. Although the theoretical value for the mobility of IR-125 could not be found, this value does correspond to the trends found for other ions.

Flow modifiers. To enhance the separations and to decrease the analysis time, electroosmotic flow modifiers were investigated. The first to be tried was the cationic surfactant dodecyltrimethylammonium bromide (DTAB). A cationic surfactant was chosen because, due to its charge, it coats the inner surface of the capillary and changes it from negative to positive. This type of surface modifier has been incorporated in other electrophoretic separations for the analysis of anions. The addition of this modifier to the IR-125 buffer system caused even more baseline drifts and instability. Upon further investigation, it was found that IR-125 forms ion-pairs with cationic surfactants.[95] This is most likely the cause of the instabilities in the fluorescence signal as well as the electroosmotic flow. Another surface modifier which has been used is diethylenetriamine (DETA). The addition of DETA also caused instability in the system.

Discussion.

While the expectations for the use of IR-125 in this CZE system were high, many problems developed during its progression. One of the most perplexing problems was the irreproducible, and unpredictable, fluorescence background. Sudden changes in the fluorescence signal along with long-term drifts and oscillations suggested an unstable electrophoretic parameters. This could have been due to the low organic content of the buffer, or some type of interaction with the IR-125 and the capillary walls.

Using a He-Cd Laser as an Excitation Source

The advantages which arise from the use of the cw helium-cadmium (He-Cd) laser as an excitation source include the many different probe species which can be utilized with indirect fluorometric detection. Unlike the large dye molecules which must be used with the diode lasers, smaller, more mobile, probe species can be used. These probes are typically water soluble, and have mobilities which are closer to those of the small inorganic anions.

Introduction to the He-Cd Laser

The He-Cd laser involves transitions of cadmium atom excited by collisions with metastable helium. The cadmium is present as a metal vapor. The He-Cd laser is a cw (continuous-wave) laser, and is pumped by an electrical discharge. The most intense wavelengths are 325 nm and 442 nm. The output power is normally around 10 mW for

the 325 nm transition, and around 15 mW for the 442 transition, but can be as high as 5000 mV. The stability of the output power is typically 0.1 %. The lifetime of the metal vapor tube is about 4000 hours. This does limit its attractiveness as an excitation source to be used for routine analysis.

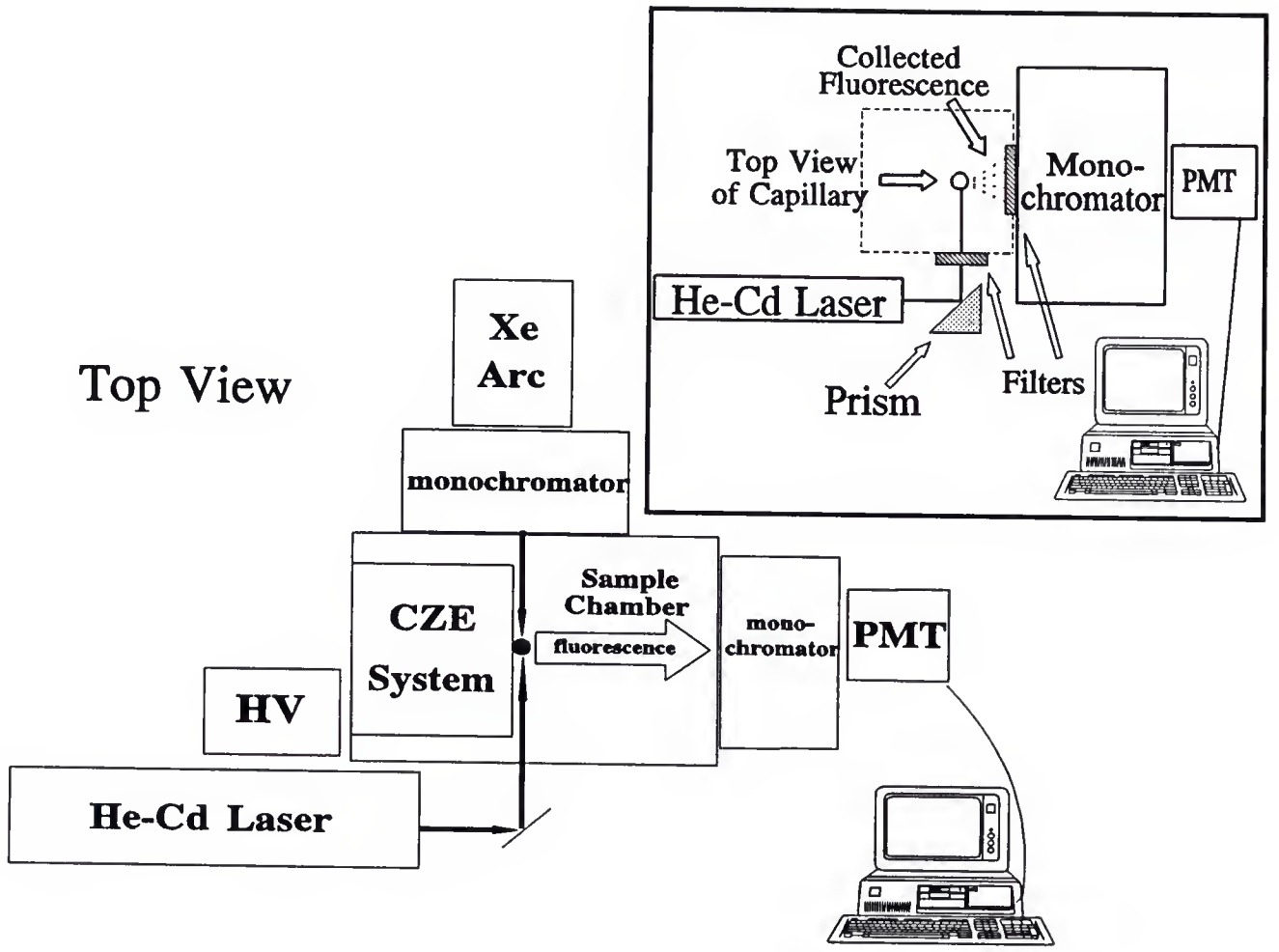
Experimental Section

Electrophoresis system. A schematic of the instrumental setup is shown in Figure 2-5. The CZE system is the same as the one used in the previous section of this chapter. One difference is that the outer diameter of the fused-silica capillary in these experiments was 360 μm, verses an outer diameter of 150 μm in the previous study. It was found that the larger outer diameter of the capillary caused no decrease in the fluorescence signal, and it was more sturdy than the capillary with the smaller outer diameter.

Excitation system. A Liconix He-Cd laser (Santa Clara,CA 94089) was used as the excitation source. The laser optics allowed use of the 325 nm line for excitation. The power output was approximately 5 mW. Several problems arose during the course of the experimentation, including the need for a new tube, and the need for the internal optics to be either cleaned, or replaced.

Detection system. On-line detection was performed, as before, by removing a small portion of the polyimide coating from the fused silica capillary. The portion of the capillary which serves as the detector window was held in the sample chamber of a SPEX Fluorolog spectrophotometer by a specially modified holder. The fluorescence was collected at 90° by a commercial 0.22 m double monochromator. A Corion P10-326

Figure 2-5: Illustration of He-Cd laser based CZE system. Inset contains schematic of instrumentation.


Collected
Fluorescence
Top View
of Capillary
Mono-
chromator
PMT
He-Cd Laser
Prism
Filters
Top View
Xe
Arc
monochromator
CZE
System
Sample
Chamber
fluorescence
mono-
chromator
PMT
HV
He-Cd Laser

cutoff filter was placed over the entrance portal of the sample chamber, to decrease the amount of unwanted stray light from entering the sample chamber. A Corion LG-370 long-pass filter, along with a piece of plexiglass, were placed before the emission monochromator to reduce the laser scatter from the capillary. It was found that the background signal produced from the capillary alone, and noise of the system could be drastically reduced by eliminating excess sources of light from reaching the detector. The emission wavelength varied slightly, depending on the probe species used, but was always around 420 nm. An R928 water-cooled PMT served as the detector. A SPEX data station and associated software were used for data collection and analysis.

Chemicals. The analyte solutions were made from the same sodium salts mentioned previously. The probe species were as follows: sodium salicylate from Sigma Corporation (St.Louis,MO 63178); sulfosalicylic acid from Kodak (Rochester,NY 14650); 5-aminoisophthalic acid from Aldrich Chemical Company (Milwaukee,WI 53233); and m-hydroxybenzoic acid from Mallincrodt (Chesterfield,MO 63017). The cationic surfactant flow modifiers were the same as mentioned previously. Barnstead nanopure water (Dubque,IA 52004) was used to make all of the solutions.

Optimization of Parameters

Probe species. Several different probe species were evaluated with this system. They are: salicylate; sulfosalicylate; m-hydroxybenzoate; and 5-aminoisophthalate. The structures of these probe species are shown in Figure 2-6. The characteristics which were considered when determining which species to investigate included the size, the

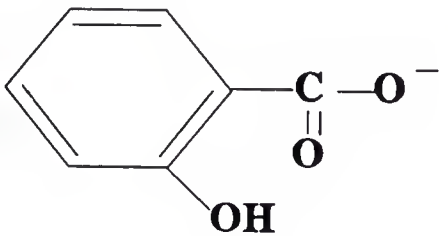
Figure 2-6: Structure of probe species for the He-Cd laser based CZE system: (a) salicylate, (b) sulfosalicylate, (c) m-hydroxybenzoate, (d) 5-aminoisophthalate.

(a)

Salicylate

$pK_1 = 2.97$

$pK_2 = 13.40$



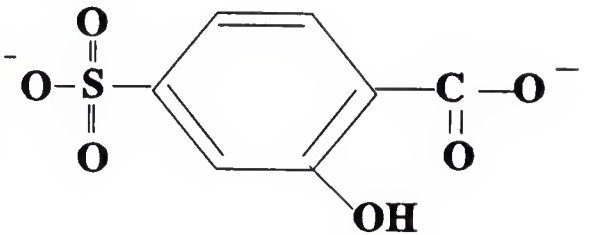
(b)

Sulfosalicylate

$pK_1 = 2.6$

$pK_2 = 2.8$

$pK_3 = 12.4$

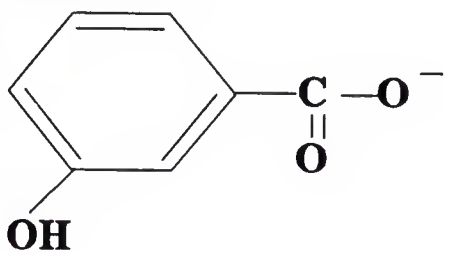


(c)

m-hydroxybenzoate

$pK_1 = 4.0$

$pK_2 = 9.9$

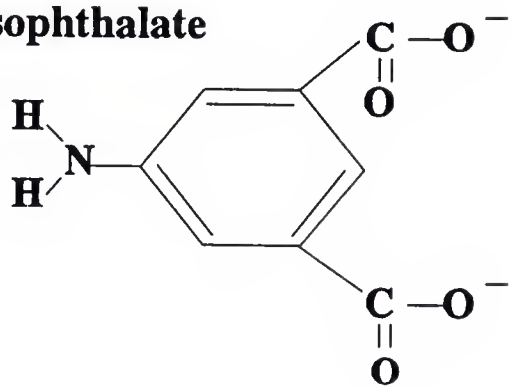


(d)

5-aminoisophthalate

$pK_1 = 3.6$

$pK_2 = 4.6$

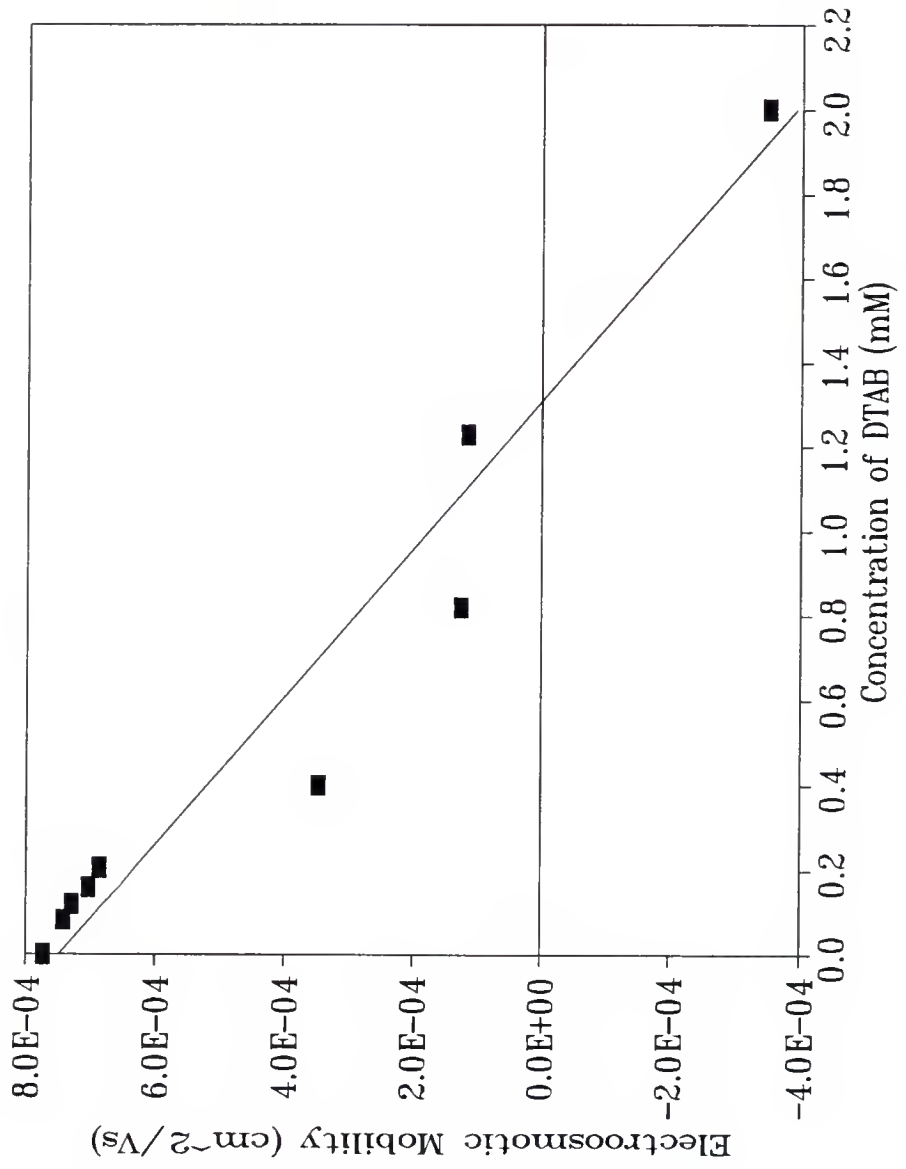


charge, the excitation wavelength, and the fluorescence intensity of the probe. Small probe species with a negative charge were desired so that the mobility could be similar to the mobility of the arsenic and selenium oxyanions. Probes with the potential of possessing a -2 charge can have higher mobilities and should also allow better displacement of the analytes, since three of the four anions also have a -2 charge. The last requirement was that the probe absorb at 325 nm, which is the output of the He-Cd laser. The fluorescence wavelength was not as crucial, as long as it was far enough away from the excitation wavelength. This allows for the scatter from the laser to be decreased by the use of filters before the emission monochromator.

Flow modifiers. As with the diode laser based system, flow modifiers were investigated to enhance the separations and to decrease the analysis time. The first to be tried was DTAB. This type of flow modifier has been incorporated in other electrophoretic separations for the analysis of anions. It was shown that the addition of varying amounts of DTAB did indeed slow down the electroosmotic flow, and eventually reverse it. The effect of this surfactant on the electroosmotic flow is shown in Figure 2-7. Two other cationic surfactants were also found to reverse the electroosmotic flow. They are: cetyltrimethylammonium bromide (CTAB); and tetradecyltrimethylammonium bromide (TTAB). TTAB was found to give the lowest fluorescence noise and the best separation.

pH. The pH of the system is important for a variety of reasons. First, the pH will determine the form of the analyte which is present, based on its pK_a values. The pH in these experiments ranged from 3 to 12, depending on the desired form of the

Figure 2-7: Effect of the surfactant DTAB on electroosmotic flow with 2.0 mM salicylate as the probe species at pH 5.3.


Electroosmotic Mobility (cm^2/Vs)
8.0E-04
6.0E-04
4.0E-04
2.0E-04
0.0E+00
-2.0E-04
-4.0E-04
0.0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
1.6
1.8
2.0
2.2
Concentration of DTAB (mM)

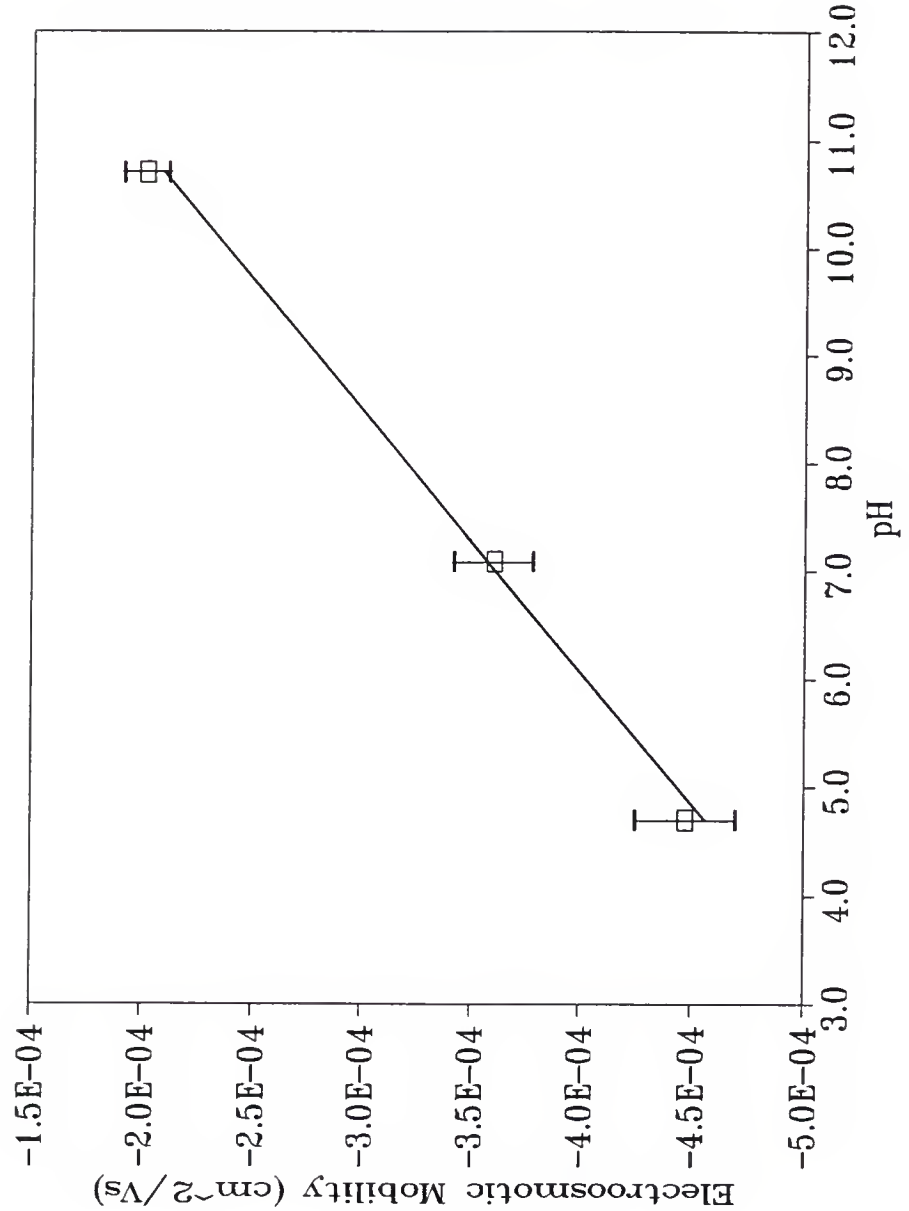
analyte or probe species. The pH also affects the electroosmotic flow of the system. Figure 2-8 shows the dependence of the electroosmotic flow on the pH. For most of the separations, a pH of 9.6 was used. This was to assure that all of the analytes were in an anionic form, so that they might be detected by a charge displacement mechanism in the indirect fluorometric detection. The other reason for the high pH was to have the probe species with a -2 charge which aided in the detection and the separation of these analytes.

Results

Salicylate probe species. Salicylate was the first probe to be used with this system. It was chosen as a test probe, since it has been utilized with CZE/IFD previously.[27-28] Initially, the system was run in "conventional" mode, with a positive power supply and injection end. With 2.0 mM salicylate at a pH of 9, μ_{eo} was 9.1 x 10^{-4} cm^2/Vs. The pH was then decreased to 3.1, and the μ_{eo} decreased to 5.1 x 10^{-4} cm^2/Vs. Neither of these conditions allowed separation or detection of the arsenic and selenium oxyanions. During this time, problems with system noise were encountered. Optimization of the emission monochromator slit widths, as well as a newly modified capillary holder were performed. It was soon after discovered that the laser was not functioning properly, and was sent in for repair. Upon its return, varying concentrations of salicylate were used as the probe species, but no effective detection was achieved.

The next step was to reverse the electroosmotic flow. The first surfactant chosen was DTAB. With this flow modifier, the electroosmotic flow was indeed reversed, and

Figure 2-8: Effect of pH on the electroosmotic flow. Buffer solution contains 2.0 mM salicylate and 2.2 mM DTAB.


Electroosmotic Mobility (cm^2/Vs)
-1.5E-04
-2.0E-04
-2.5E-04
-3.0E-04
-3.5E-04
-4.0E-04
-4.5E-04
-5.0E-04
3.0
4.0
5.0
6.0
7.0
8.0
9.0
10.0
11.0
12.0
pH

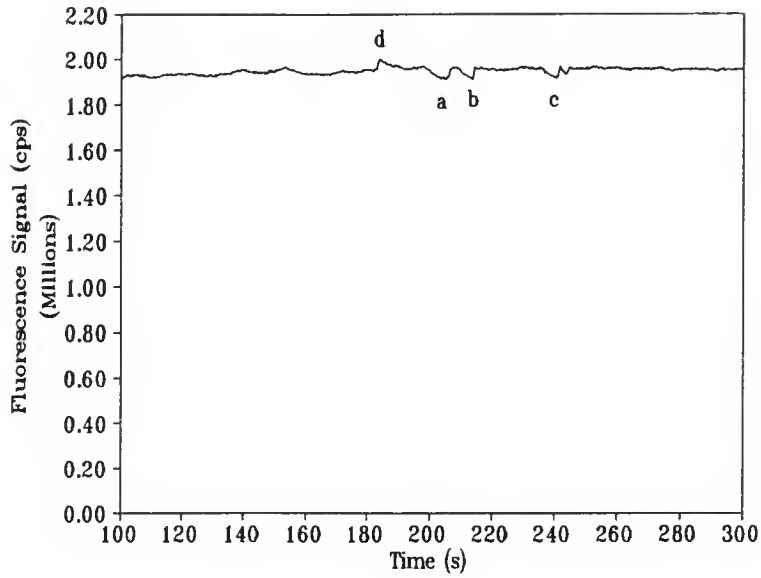
separation of the oxyanions was attempted. The results, unfortunately, were erratic. The fluorescence baseline would change irregularly, and the noise would also change without warning; this occurred with varying concentrations of salicylate (although it was more common at the lower salicylate concentrations), with a DTAB concentration of 2.0 mM. The DTAB was kept at 2.0 mM to facilitate reversal of the electroosmotic flow.

After experiencing difficulty with the DTAB, a second surfactant, TTAB, was tried. A buffer solution containing 2.0 mM salicylate and 0.5 mM TTAB at a pH of 9.7 showed promising results. Figure 2-9 shows an electropherogram in which $HAsO_4^{-2}$ and SeO_3^{-2} were separated. The signal-to-noise ratio (DR) for this system was calculated to be 775. This is in the range of expected values for excitation with a He-Cd laser. The μ_{eo} of this system was found to be -3.5 x 10^{-4} cm^2/Vs. The individual mobilities were calculated as the following: SeO_4^{-2} -6.2 x 10^{-4} cm^2/Vs; SeO_3^{-2} -5.7 x 10^{-4} cm^2/Vs; $HAsO_4^{-2}$ -5.3 x 10^{-4} cm^2/Vs; and AsO_2^{-1} -2.8 x 10^{-4} cm^2/Vs. In an attempt to lower the detection limits (which were in the ppm range), a lower concentration of salicylate was evaluated. This did not, however, help the separation or the detection. It was detrimental to the detection of the same concentrations of analytes used above, as well as to the signal-to-noise ratio of the system. The signal-to-noise ratio (DR) dropped to 490, from 775. It appears that the separation and detection of these four analytes is possible with salicylate as the probe molecule, but not ideal. Further optimization of the separation parameters was not performed.

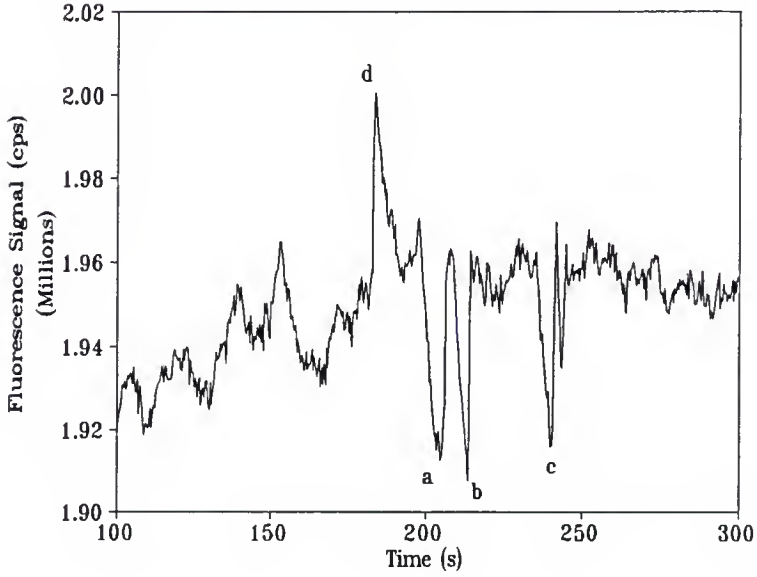
5-aminoisophthalate. The next probe which was tried was 5-aminoisophthalate (AIP). While this probe did have a -2 charge and it was highly fluorescent, it was not

Figure 2-9: Electropherogram of arsenic/selenium oxyanion mixture with buffer containing 2 mM salicylate and 0.5 mM TTAB at pH 9.7 at -20 kV. (A) full scale view; (B) magnified view. Signals are designated as: (a) 13 ppm SeO_3^{-2}; (b) 14 ppm $HAsO_4^{-2}$; (c) salicylate (probe); (d) possible impurity.

A


Fluorescence Signal (cps)
(Millions)
2.20
2.00
1.80
1.60
1.40
1.20
1.00
0.80
0.60
0.40
0.20
0.00
d
a
b
c
100
120
140
160
180
200
220
240
260
280
300
Time (s)

B

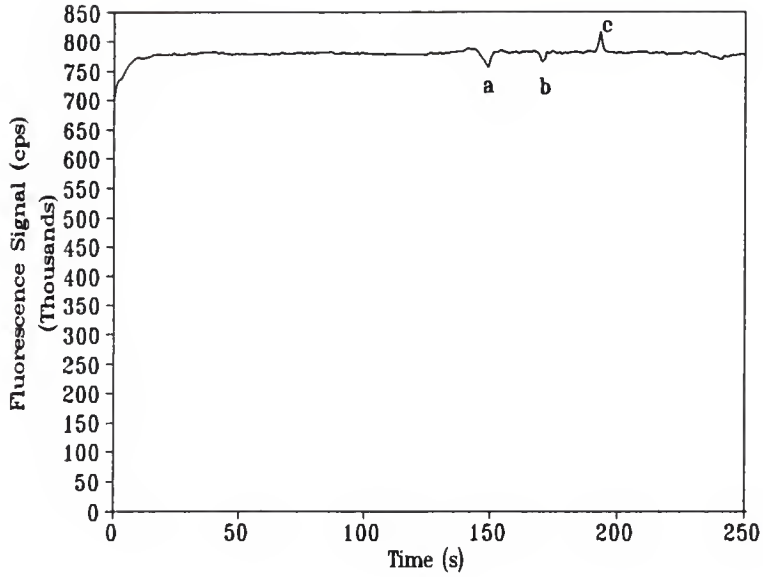

Fluorescence Signal (cps)
(Millions)
2.02
2.00
1.98
1.96
1.94
1.92
1.90
d
a
b
c
100
150
200
250
300
Time (s)

very water soluble. The most concentrated solution which could be made was 0.25 mM. With 0.25 mM AIP and 0.5 mM TTAB, a signal-to-noise (DR) of 1220. While the baseline was very stable before an injection, the baseline became erratic a few minutes into the run and then it re-stabilized. It is suspected that the amino group on the probe has a detrimental effect on the indirect fluorometric detection (and possible on the separation as well). Occasionally sharp dips in response were observed, but the large drifts in the background signal hampered thorough evaluation of these dips.

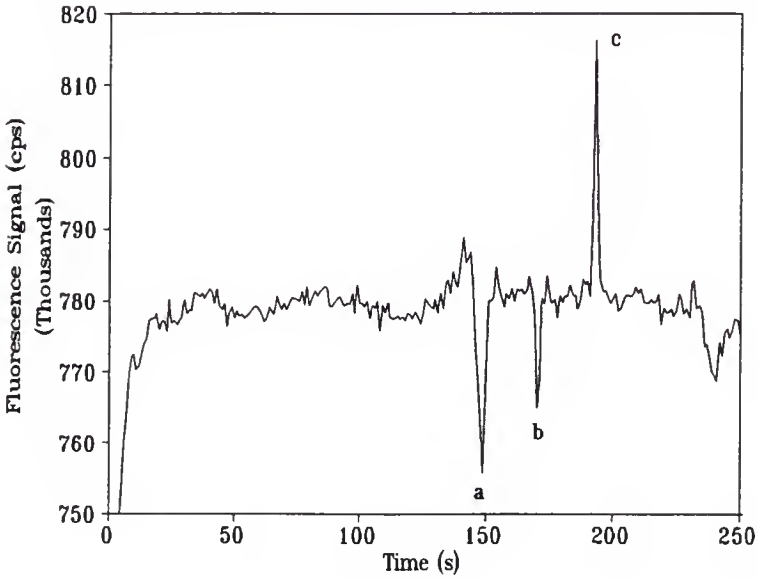
Sulfosalicylate. With the difficulties encountered by the use of salicylate and AIP as probes, sulfosalicylate was evaluated as a probe. Sulfosalicylate (SS) is also excited by the 325 nm line of the He-Cd laser, and has a fluorescence at 422 nm. Again, varying concentrations of sulfosalicylate were evaluated (from 1.0 mM to 0.1 mM), with TTAB as the flow modifier. Figure 2-10 shows a separation of SeO_4^{-2} and SeO_3^{-2} with a buffer consisting of 1.0 mM sulfosalicylate and 0.5 mM TTAB at pH 9.7. The electroosmotic mobility was -2.0×10^{-4} cm^2/Vs. The mobilities of two of the analytes were as follows: SeO_4^{-2} -7.0×10^{-4} cm^2/Vs; SeO_3^{-2} -5.8×10^{-4} cm^2/Vs. The signal-to-noise ratio (DR) for this system was 953, which is in the same range as the other probes evaluated with the He-Cd laser based system. When a mixture of all four analytes was injected, the background fluorescence signal became noisy at times, which hampered the simultaneous separation of the arsenic and selenium oxyanions with this probe. As with the salicylate, decreasing the concentration of the probe below 1.0 mM caused further baseline instability, and no improvement in the signals was observed when the anions were injected.

Figure 2-10: Electropherogram of arsenic/selenium oxyanion mixture with a buffer containing 1.0 mM sulfosalicylate and 0.5 mM TTAB at pH 9.7 at -30 kV: (A) full scale view; (B) magnified view. Signals are designated as: (a) 14 ppm SeO_4^{-2}, (b) 13 ppm SeO_3^{-2},(c) sulfosalicylate (probe).

A


Fluorescence Signal (cps)
(Thousands)
850
800
750
700
650
600
550
500
450
400
350
300
250
200
150
100
50
0
0
50
100
150
200
250
Time (s)
a
b
c

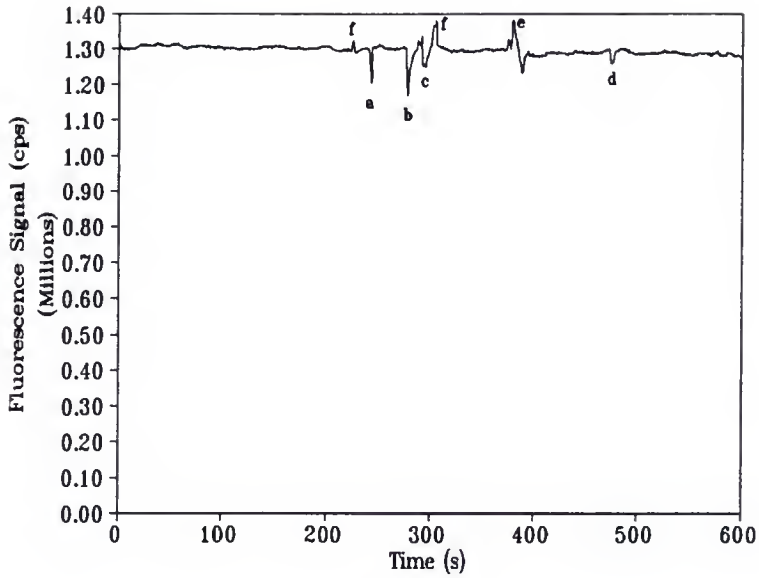
B


Fluorescence Signal (cps)
(Thousands)
820
810
800
790
780
770
760
750
0
50
100
150
200
250
Time (s)
a
b
c

m-hydroxybenzoate. The last probe which was investigated was m-hydroxybenzoate. The difference between this probe and the salicylate was simply the location of the hydroxide group on the benzene ring. As with the others, varying concentrations of this probe were evaluated. It was found that concentrations lower than 1.0 mM of m-hydroxybenzoate did not allow effective analysis of the arsenic nd selenium oxyanions. At a buffer composition of 1.0 mM m-hydroxybenzoate and 0.5 mM TTAB at pH 9.9, analysis of all four anions was indeed possible. Figure 2-11 shows this separation. The mobilities for the analytes are as follows: SeO_4^{-2} -7.0 x 10^{-4} cm^2/Vs; SeO_3^{-2} -6.0 x 10^{-4} cm^2/Vs; $HAsO_4^{-2}$ -5.7 x 10^{-4} cm^2/Vs; and AsO_2^{-1} -3.0 x 10^{-4} cm^2/Vs. The μ_{eo} of the system was measured to be -1.3 x 10^{-4} cm^2/Vs. The signal-to-noise ratio (DR) was 892. Figures 2-12 through 2-16 show the calibration curves for these analytes, as well as the calibration curve for m-hydroxybenzoate. The limits of detection for this system are given in Table 2-2 and are based on the area of the dip observed. The linear dynamic range for this system was around 1 order of magnitude. Part of the reason for this was the limitations placed on the concentration of the analyte in the sample by the concentration of the buffer solution. The best resolution should occur when the analyte-to-buffer ratio is 1:500. When the analyte concentration reached that of the buffer solution, the injected zone had a higher resistance than the buffer solution, hence a lower current flowing through it. This caused it to move through the capillary as a plug, rather than separating into the corresponding analyte zones. The lower end of the concentration scale was determined by the limit of detection for the system. The transfer ratios for these analytes were calculated as follows: SeO_4^{-2} 0.32; SeO_3^{-2} 0.46; $HAsO_4^{-2}$ 0.34; and

Figure 2-11: Electropherogram of arsenic/selenium oxyanion mixture with buffer containing 1.0 mM M-hydroxybenzoate and 0.5 mM TTAB at pH 9.9 at -20 kV: (A) full scale view; (B) magnified view. Signals are designated as: (a) 14 ppm SeO_4^{-2}; (b) 13 ppm SeO_3^{-2}; (c) 14 ppm $HAsO_4^{-2}$; (d) 11 ppm AsO_2^{-1}; (e) m-hydroxybenzoate (probe); (f) unidentified.

A


Fluorescence Signal (cps)
(Millions)
1.40
1.30
1.20
1.10
1.00
0.90
0.80
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0.00
0
100
200
300
400
500
600
Time (s)
a
b
c
d
e
f
f

B

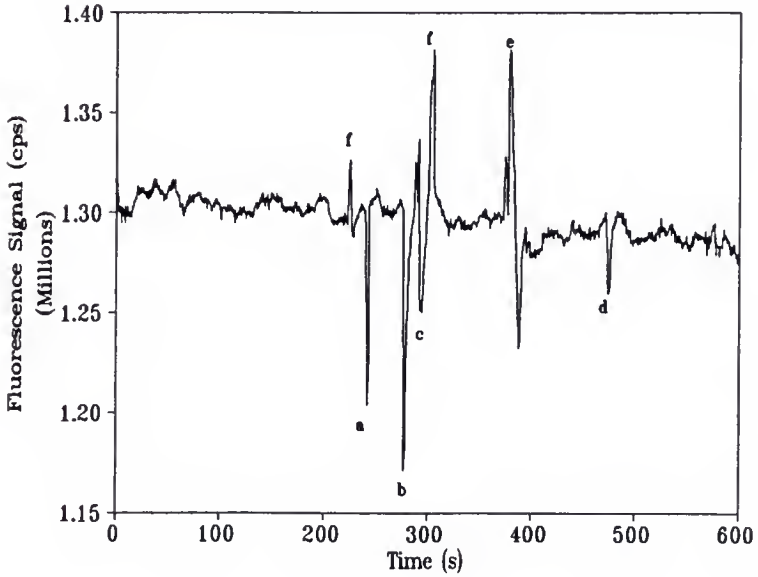

Fluorescence Signal (cps)
(Millions)
1.40
1.35
1.30
1.25
1.20
1.15
0
100
200
300
400
500
600
Time (s)
a
b
c
d
e
f
f

Figure 2-12: Analytical calibration curve for SeO_4^{-2} using a buffer of 1.0 mM m-hydroxybenzoate and 0.5 mM TTAB at pH 9.9. ($Y = 2.36 \times 10^{17} X - 6.48 \times 10^{3}$; $R = 0.9972$).

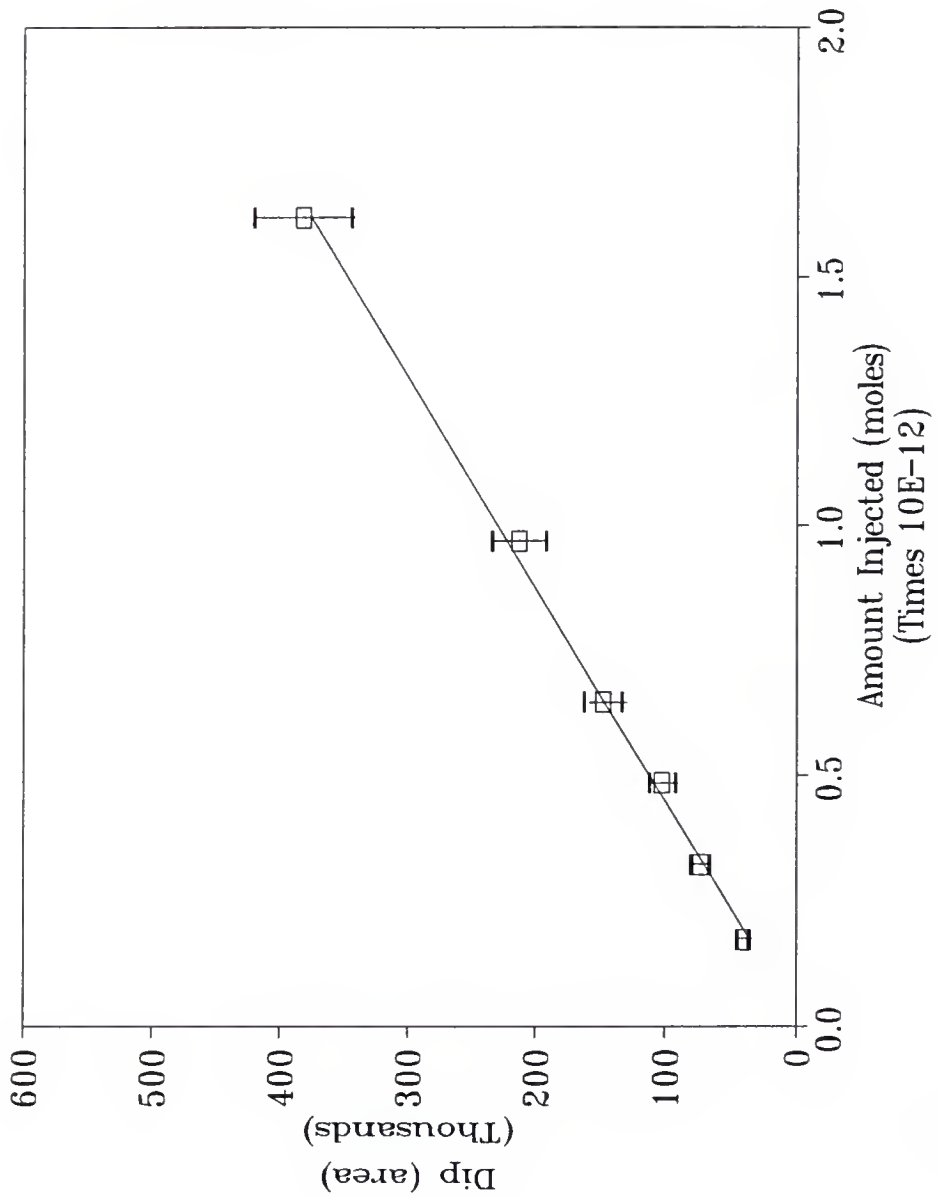

Dip (area)
(Thousands)
600
500
400
300
200
100
0
0.0
0.5
1.0
1.5
2.0
Amount Injected (moles)
(Times 10E-12)

Figure 2-13: Analytical calibration curve for SeO_3^{-2} with buffer of 1.0 mM m-hydroxybenzoate and 0.5 mM TTAB at pH 9.9. ($Y = 1.29 \times 10^{17} X + 1.08 \times 10^{4}$; $R = 0.9837$)

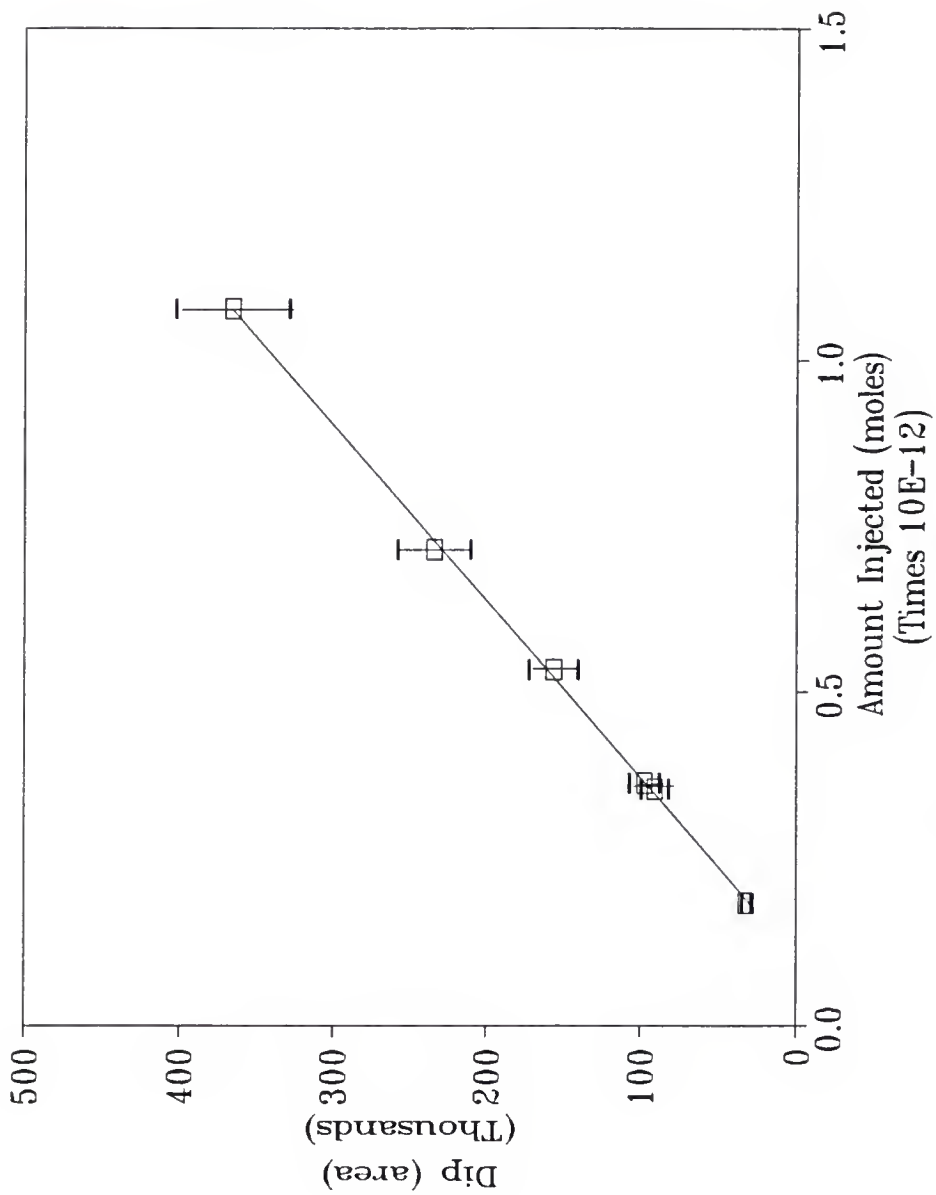

Dip (area)
(Thousands)
500
400
300
200
100
0
0.0
0.5
1.0
1.5
Amount Injected (moles)
(Times 10E-12)

Figure 2-14: Analytical calibration curve of $HAsO_4^{-2}$ with buffer of 1.0 mM m-hydroxybenzoate and 0.5 mM TTAB at pH 9.9.
($Y = 2.78 \times 10^{17} X - 2.92 \times 10^{4}$; $R = 0.9932$)

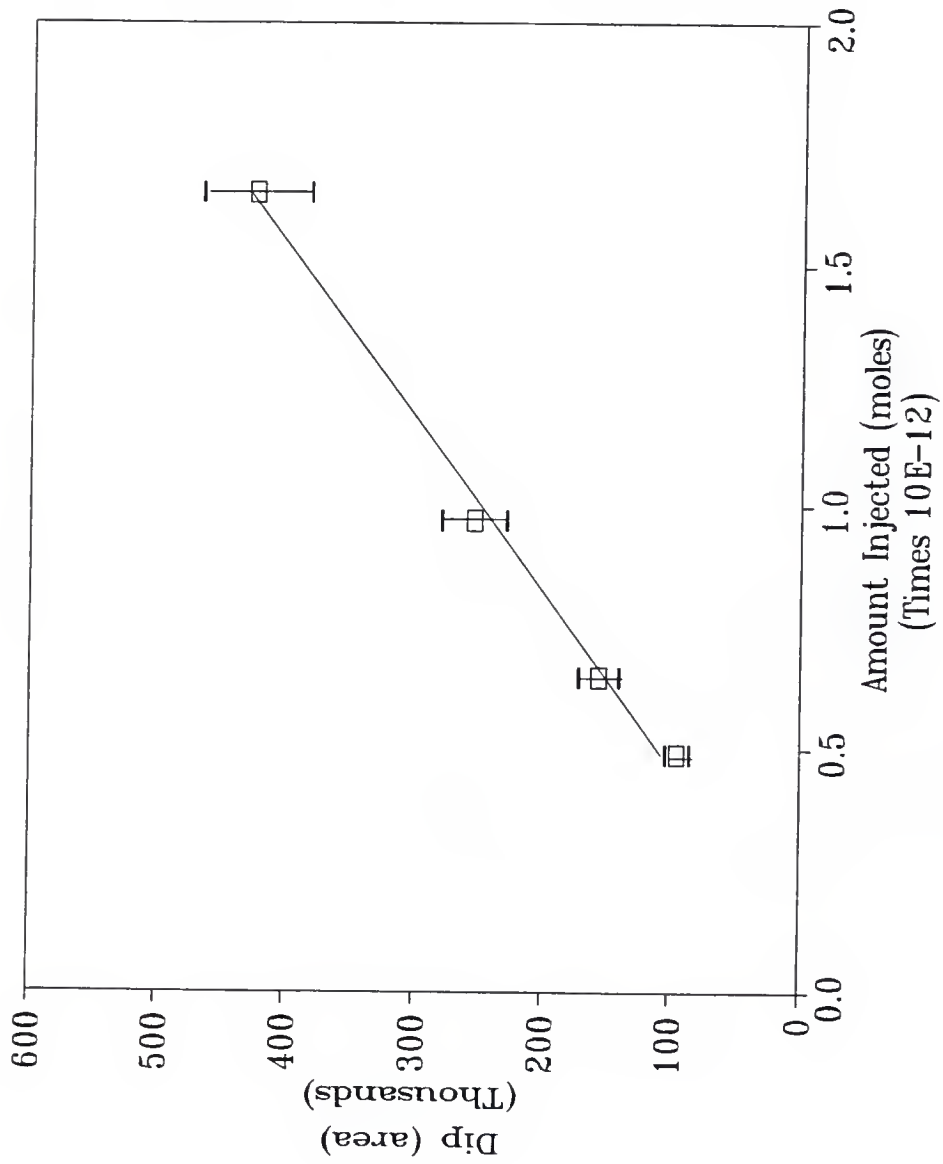

Dip (area)
(Thousands)
600
500
400
300
200
100
0
0.0
0.5
1.0
1.5
2.0
Amount Injected (moles)
(Times 10E-12)

Figure 2-15: Analytical calibration curve for AsO_2^{-1} with buffer of 1.0 mM m-hydroxybenzoate and 0.5 mM TTAB at pH 9.9.
($Y = 6.57 \times 10^{16} X - 6.00 \times 10^{3}$; $R = 0.9830$)

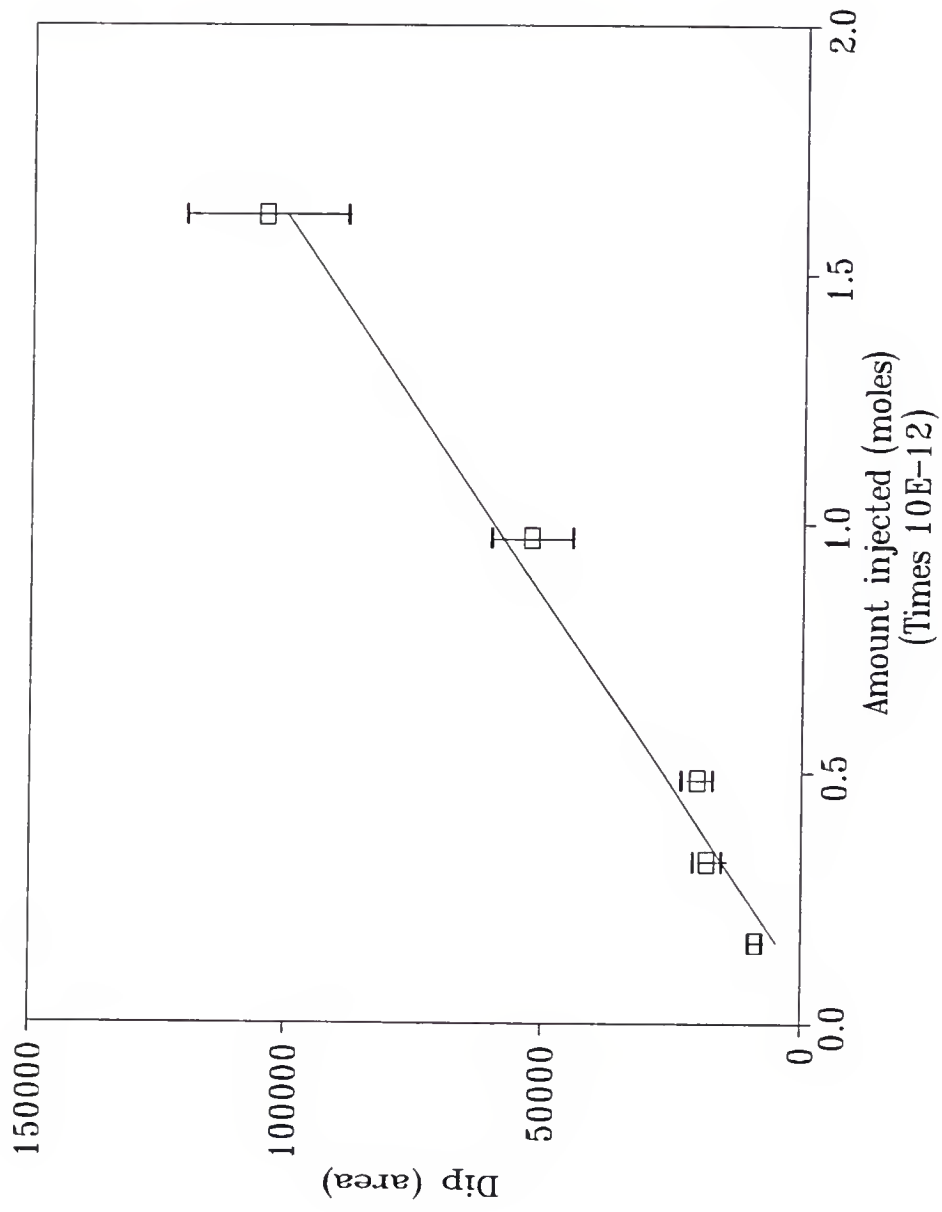

150000
100000
50000
0
Dip (area)
0.0
0.5
1.0
1.5
2.0
Amount injected (moles)
(Times 10E-12)

Figure 2-16: Analytical calibration curve for m-hydroxybenzoate. Buffer solution contains 50 mM carbonate at pH 9.6 at -30 kV.

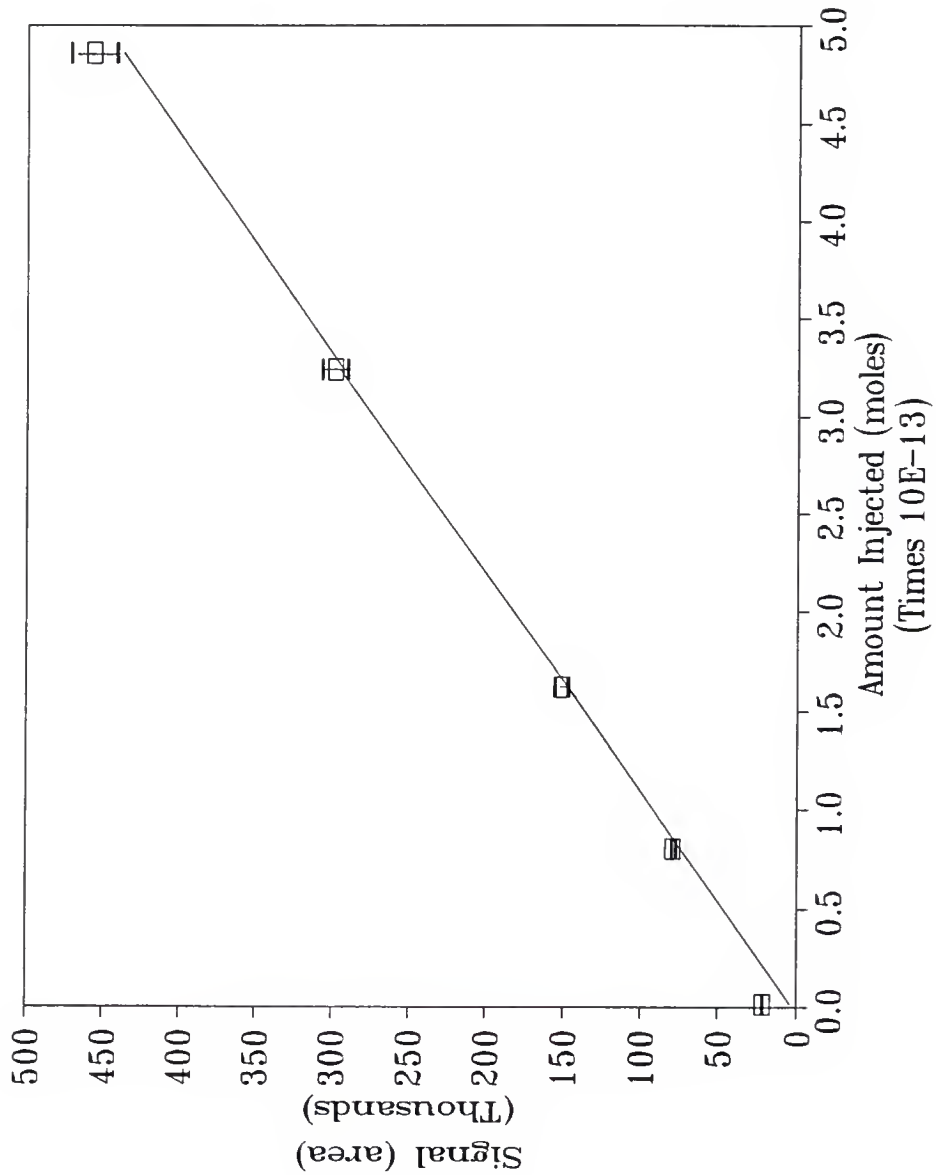

Signal (area)
(Thousands)
500
450
400
350
300
250
200
150
100
50
0
0.0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
4.0
4.5
5.0
Amount Injected (moles)
(Times 10E-13)

Table 2-2: Limits of detection using the CZE/He-Cd laser based system with m-hydroxybenzoate as the probe species.

Analyte	CLOD[a]	CLOD[b]	MLOD[c]
SeO_4^{-2}	4.5	0.64	12
SeO_3^{-2}	3.0	0.38	8
$HAsO_4^{-2}$	24.0	3.4	63
AsO_2^{-1}	4.2	0.45	10

[a] concentration limit of detection in μmol/L.
[b] concentration limit of detection in ppm.
[c] absolute limit of detection in fmol.

AsO_2^{-1} 0.06. Similar values for the three anions with -2 charges suggested that a charge displacement of the -2 charged probe species was occurring. The arsenite anion had a lower transfer ratio, since the displacement of the probe by the anion was not as strong.

Discussion.

These experimental results have shown that the analysis of arsenic and selenium oxyanions can be accomplished using CZE with indirect fluorometric detection. With m-hydroxybenzoate as the probe species, separation of these analytes was possible in less than 10 minutes. The detection limits for this system were comparable to those obtained by indirect UV-vis absorption detection. One of the problems which prevented lower detection limits from being achieved had to do with the collection optics. The SPEX system was designed to collect the fluorescence from a cuvette which is placed in the

sample chamber. If the capillary was not placed in the exact location necessary for optimum fluorescence collection, a loss of sensitivity was observed. While this optimization was carefully performed with the fluorescent probe in the capillary, the different positions of the capillary also changed the "blank" signal, ie. the signal observed when no fluorescent probe was present. This made optimization more complicated. Another possible source of limited sensitivity may have arisen from the He-Cd laser noise. Addition of a laser power stabilizer could enhance the detection limits by increasing the signal-to-noise ratio of the system.

CHAPTER 3
ION CHROMATOGRAPHY WITH INDIRECT FLUOROMETRIC DETECTION

Theoretical Aspects of Ion Chromatography

Ion chromatography (IC) is a form of high performance liquid chromatography (HPLC) which is used for the separation of ionic compounds. This separation technique combines chromatography with ion-exchange. Determination of exactly when the two techniques were combined are debatable, but an important landmark was the ion-exchange work of Adams and Holmes in 1935.[95] From that time until the early 1970s, the theory of ion-exchange chromatography, as well as its applications, were investigated. With the development of automated detectors and advances in separation, the use of ion-exchange chromatography blossomed. The types of analytes which were being separated and detected included: amino acids, rare earth elements, proteins, dyes, pharmaceuticals, synthetic polymers, and some inorganic ions. At this point in time, the most common detector was the UV absorption detector.[95] In the mid 1970s, Dow Chemical Company granted a license to Dionex Corporation to manufacture and market instrumentation which utilized suppressed conductivity detection for ion exchange chromatography. They called this technique "ion chromatography", and applied it to the analysis of many inorganic and organic ions which were undetectable by UV absorption or refractive index detectors.[95] Today, ion chromatography is applied to a wide variety of ionic species in

all types of matrices. The detectors which can be used with IC are also expanding, and include: UV absorbance,[96] indirect UV absorption,[12,21,22,97-100] and indirect fluorescence.[37,38,101]

Definitions

A basic IC system consists of a pump, and injection valve, a separator column, and a detector. The main difference between HPLC and IC is the stationary phase in the separation column. The mobile phase (eluent) of the system serves only to transport the analytes through the chromatographic system. The separation occurs due to the different affinities of the analytes for the stationary phase, which is an ion-exchanger.

The rate at which an analyte moves through the chromatographic system is determined by its distribution constant, K, between the two phases:

$$K = \frac{C_S}{C_M} \tag{3-1}$$

where C_S is the concentration of solute (analyte) in the stationary phase (mol/L), and C_M is the concentration of solute in the mobile phase (mol\L). The capacity factor, k, is given by the ratio of the number of molecules in the stationary phase, n_S, to those in the mobile phase ,n_M:

$$k = \frac{n_S}{n_M} = \frac{C_S V_S}{C_M V_M} = K\frac{V_S}{V_M} \tag{3-2}$$

where V_S is the volume of the stationary phase (L), and V_M is the volume of the mobile phase (L).

The volume of eluent which is required to remove all of the analyte from the column is called the total retention volume, V_R. The total free volume of the system, including the injection loop, the column, and all connection tubing is called the "dead" volume and is determined by the retention time of an unabsorbed analyte. The actual retention volume of the analyte is given by:

$$V_R' = V_R - V_o \tag{3-3}$$

where V'_R is the corrected retention volume (L), and V_o is the dead volume (L). Since chromatograms are typically recorded as a function of time, it is the retention time, t_R, which is determined. From the t_R (s) and the flow rate, F (L/s), the retention volume (L) can be determined by the relationship:

$$V_R = t_R F \tag{3-4}$$

The capacity factor can be related to the retention time or the retention volume by the equation:

$$k = \frac{V_R'}{V_o} = \frac{t_R'}{t_o} \tag{3-5}$$

The separation of two analytes is determined by their distribution constants, and can be defined as a separation factor, α:

$$\alpha = \frac{k_2}{k_1} = \frac{K_2}{K_1} \tag{3-6}$$

The efficiency of the separation is defined by the number of theoretical plates, which can be defined as:

$$N = \frac{L}{h} = 5.54 \left(\frac{t_R}{w_h} \right)^2 \qquad (3\text{-}7)$$

where L is the length of the column (cm), h is the height equivalent to a theoretical plate (HETP) (cm), and w_h is the width of the peak at half of its height (s). The resolution of the chromatographic process is defined as:

$$R_S = \frac{t_{R2} - t_{R1}}{\frac{1}{2}(w_{b2} + w_{b1})} \qquad (3\text{-}8)$$

where w_b is the width of the peak at the base (s). The resolution of a separation can be related to the capacity factor, and the separation factor by the equation:

$$R_S = \frac{1}{4} \sqrt{N} \ (\alpha - 1) \left(\frac{k}{1+k} \right) \qquad (3\text{-}9)$$

This equation is a fundamental relationship in chromatography and clearly indicates how N, α, and k can affect the separation.

As was mentioned, the stationary phase in IC is an ion exchanger. The success of IC as a separation technique depends on the relative affinities of the exchangeable (analyte) ions toward the fixed (stationary phase) ions, and the co-ion (solute). The theory of this affinity is not completely understood. It does, however, depend on the electrostatic field strength around the fixed ions and the radius of the spherical (analyte) ions. Solvation of the ions by the solvent also exerts considerable influence on the electrostatic interactions. In general, the affinity of the ions for the stationary phase is

inversely proportional to the radius of the hydrated ion and directly proportional to the ionic charge.[102]

Ion Exchangers

The key to an IC separation is the stationary phase. This determines the separation mechanism, and the separating ability of the entire system. There are 3 important elements which make up an ion exchange stationary phase. They are: an insoluble matrix, which can be organic or inorganic; the fixed ionic sites, which are attached to or a part of the matrix; and the equivalent number of ions of charge opposite to that of the fixed sites. The attached groups are often called functional groups, and the associated ions are called counterions. Other desired characteristics for an IC stationary phase include the ability to exchange ions rapidly, chemical stability over a large pH range, mechanical stability, and resistance to deformation during packing and when subjected to the flow of the mobile phase.[95]

Modern IC ion exchangers use 2 major matrices: silica and organic polymers based on styrene. The most common way to produce an ion exchanger is to make a styrene-based polymer and chemically modify the polymer to introduce the functional groups. One type of organic polymer matrix is the gel-type polymer. The degree of swelling depends on the amount of divinyl benzene (DVB) present. The DVB forms crosslinking in the polymer. The degree of crosslinking is given as mole percent DVB. These types of resins will readily admit small ions and molecules, but resist the intrusion of larger species. Lower crosslinking improves the diffusion through the resin. Practical

limits on crosslinking are usually in the range of 4% to 12%.[95] The most common functional group for anion analysis is the quaternary amine group. A typical procedure for low-capacity ion exchangers is the surface agglomeration method. With this method the surface sulfonated styrene-DVB copolymer particles are contacted with colloidal anion exchange particles. Resins produced by this method are called pellicular resins. Pellicular resins have the advantages of an increased resistance to higher pressures, stability in broader pH range, and stability in the presence of organic solvents. They also produce a reduction of the diffusion path, which accelerates exchange of the eluent and analyte ions which increases the separation efficiency.

Eluents

A particular eluent is considered to be the optimum choice if it gives rapid, selective and sensitive determination of the analyte ions. The first consideration is the composition of the eluent. As a general rule, the retention properties of the eluent and the analyte ions should be similar. For example, for the determination of weakly retained analyte ions, a weakly retained eluent should be used. For the analysis of a mixture of weakly and strongly retained ions, it is sometimes possible to use one eluent to achieve an adequate separation. If not, the other option is to use gradient elution to change the strength of the eluent so as to separate the analytes, and also decrease the analysis time. Many different types of eluents have been incorporated into IC separations, including: hydroxide, carbonate, bicarbonate, aminoacetate, glycinate, benzoate, tartrate, oxalate, salicylate, aminosalicylate, and phthalate.[101]

The concentration of the eluent can be varied and optimized for a particular separation. Variation of the concentration of the eluent changes the ion-exchange equilibrium. Increasing the eluent concentration causes an increase in the eluting power of the eluent. The pH of the system can also be used to control the selectivity of the separation. An increase in pH can shift the equilibrium of the eluent species to produce an anion with a larger negative charge and an increase in eluting power. Organic additives can be added to the eluent to improve the separation of some ions. The addition of acetonitrile tends to increase the eluting power and therefore decrease the analysis time. Methanol has also been used as an organic additive.[100]

An ion chromatogram may have two extra peaks. The first of these is called the water, or injection peak, which is a result of the passage of the unretained zone of water, containing the eluting anion in an amount equivalent to the anionic composition of the sample.[103] The presence of this peak can hamper the analysis of anions which are very weakly retained. The "dead volume" of the system can be determined by the retention time of this peak. In a dual-column system (one which has a suppressor column as well as a separation column), this peak can be reduced by using demineralized water, or eluents which give a low signal at the detector. In a single-column system, there is no effective way to eliminate this peak. The second "extra" peak is called the system peak. It occurs due to the desorption of the molecular form of the eluent from the separator column on sample injection. The system peak usually occurs at long retention times, and can interfere with the analysis of some analytes. The time, height and area of this peak depends on the pH, the concentration, and the volume of the injected sample. The most

efficient way to eliminate the second extra peak is to use eluents at high pH values so as to not have any molecular form of the eluent present.[103]

Detection Methods

A wide variety of detection methods have been used with IC. Because of the desire to achieve lower detection limits, the evaluation of new detection methods for IC continues. Detectors for IC must be: highly sensitive, have a small cell volume, have a highly stable baseline signal, and the signal should remain stable when the flow-rate of the eluent is changed.

Conductometric detection. Conductometric detection for IC sounds like an ideal situation, since each ion will produce a signal at the detector. However, this detection technique was hampered by the high ionic strength of the eluent itself. It was not until the early 1970s when ion suppression was developed, that conductometric detection for IC became a viable detection method.[104] In this case, the eluent is converted into a low-conductivity compound, thus decreasing the background signal. Dionex Corporation has developed a membrane suppressor (AMMS) which allows very efficient ion suppression, allowing even gradient elution to be used with their system. Single-column IC with conductivity detection has been evaluated by the use of organic acids as eluents.[105,106] It is, however, the suppressed IC technique which has allowed IC to become a more common analytical technique for the analysis of ions.

Spectroscopic detection. The first spectroscopic detectors used for IC were UV-visible absorption detectors. This can be used for the determination of organic ions,

especially those of aromatic compounds which absorb strongly in the UV region of the spectrum. Direct UV absorption can be a more sensitive and selective detection method for many ions.[103] For those analytes which do not absorb, one option is to perform a post-column reaction which will produce a species which will produce a signal at the detector. This type of analysis is not all that common, but has been used to determine rare earth metals and transition metals.[103]

Indirect detection methods. Indirect photometric chromatography was first proposed by Small and Miller in 1982.[12] Because of its sensitivity and universal nature, indirect photometric detection (IPD) has been utilized for those ions for which conductivity or direct UV absorption could not be used.[21,22,97-100] One reason IPD has grown so rapidly is that many of the eluents already being used for IC with conductivity detection, could be used with this detector. Many times, conductivity and IPD are used in conjunction with each other to provide complete analysis of a sample. The technique of indirect fluorometric detection (IFD) has also been used with IC.[37,38,101] Since fluorescence is inherently a more sensitive technique than absorption, IFD can extend this to species which do not themselves fluoresce. This technique has been used to detect common inorganic anions at the picogram level.[101]

Using a Diode Laser as an Excitation Source

As was the case for the CZE separations, the use of a diode laser for excitation in IC was evaluated.

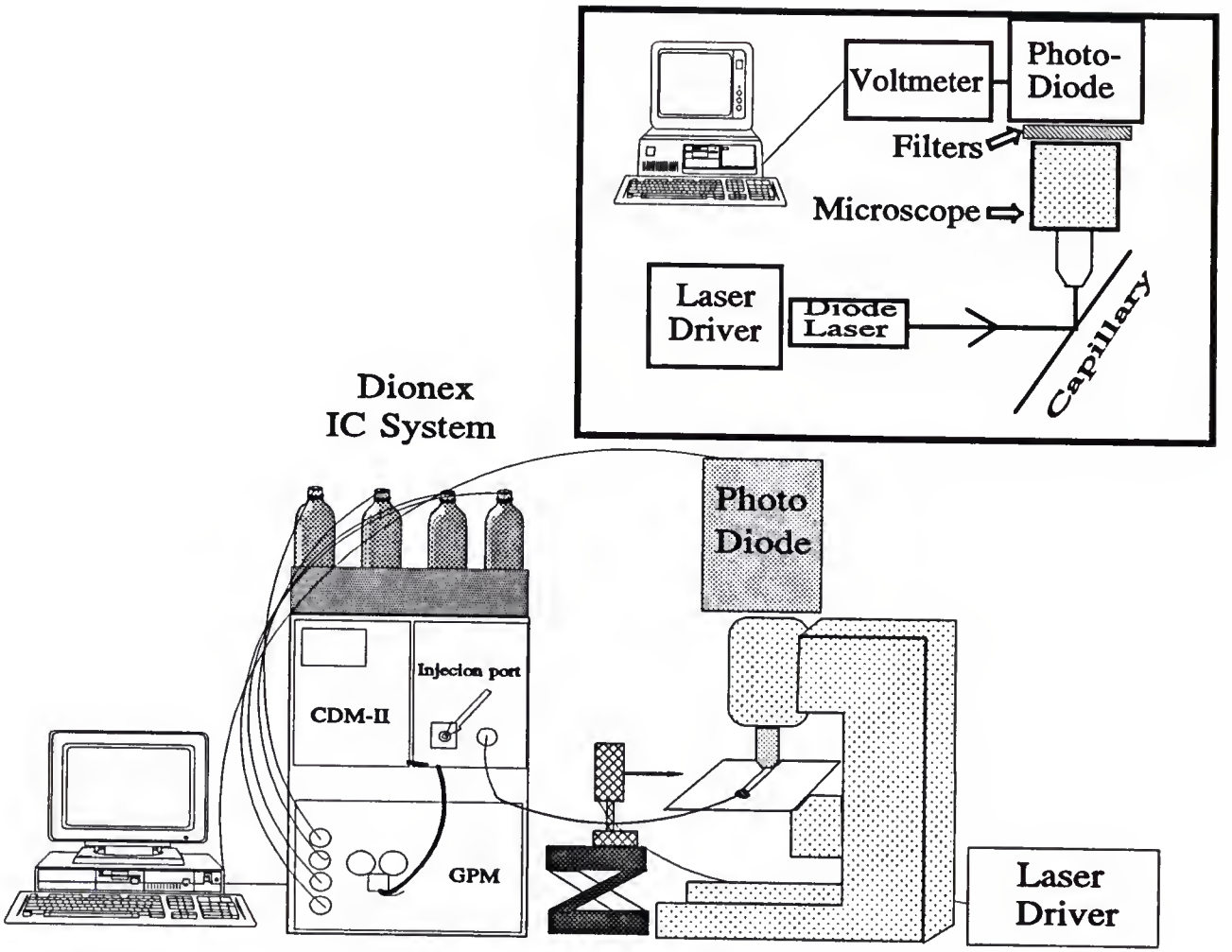
Experimental Section

Chromatography system. A schematic of the instrumental setup is shown in Figure 3-1. It consisted of a Dionex Series 4500 Chromatography system. This system utilized an eluent degas module in conjunction with a gradient pump module which allowed mixture of 4 different eluents, as well as gradient elution programs. A 50 μL injection loop was used throughout. An AG-11 guard column preceded and AS-11 anion separator column. A Dionex Advanced Computer Interface was used to interface the IC system with a computer for data collection and analysis.

Excitation system. A Mitsubishi diode laser driven by a Spectra Diodes SDL 800 laser driver and an ILX LDT-5910 thermoelectric temperature controller was used as the excitation source. The excitation wavelength of the laser was 780 nm at 20° C and 49 mA of current. The output power was 3 mW.

Detection system. A CDM-II conductivity detector, which was part of the Dionex IC system (Sunnyvale,CA 94088) was used as a comparison to the indirect fluorometric detector which is described below. An AMMS-II membrane suppressor was used when suppressed conductivity detection was desired. The diode laser based system followed the conductivity detector. A flow cell was made from a section of Polymicro Technologies (Phoenix,AZ 85017) fused silica capillary about 8 cm long. The outer diameter of the capillary was 360 μm, and the inner diameter was 250 μm. The calculated volume excited by the laser was 10 nL. This flow cell was held on a microscope stage by a specially designed holder. The fluorescence was collected at 90° by a Nikon 20x microscope objective. Two cutoff filers were placed before the detector

Figure 3-1: Illustration of diode laser-based system. Inset contains schematic of the instrumentation.


Voltmeter
Photo-
Diode
Filters
Microscope
Laser
Driver
Diode
Laser
Capillary
Dionex
IC System
Photo
Diode
Injecion port
CDM-II
GPM
Laser
Driver

to reduce laser scatter from the flow cell. One was a Corion RG 850 high-pass filter (Holliston,MA 01746), and the other was a Kodak #87C Wratten filter (Rochester,NY 14650). The detector was a Hamamatsu HC210-3314 red-sensitive photodiode (Bridgewater,NJ 08807) with amplifier. The output was directed to a Keithley 182 Sensitive Voltmeter (Cleveland,OH 44139), which was interfaced to the Dionex IC software. This software allowed for simultaneous conductivity and IFD detection.

Chemicals. The analyte solutions were made from their sodium salts. Sodium arsenite, sodium arsenate, sodium selenite, and sodium selenate were obtained from Sigma Corporation (St.Louis,MO 63178). Standard anion solutions of fluoride, chloride, bromide, nitrite, nitrate, and sulfate were made from 200 ppm standards obtained from Alltech Associates Inc. The near-IR dye, IR-125, was obtained from Exciton Corporation (Dayton,OH 45431). Sodium hydroxide was made from 50% solutions obtained from Fisher Scientific (Pittsburgh,PA 15219). The pH was adjusted, when necessary, with a dilute solution of sodium hydroxide. Barnstead nanopure water (Dubuque,Iowa 52004) was used to prepare all of the solutions. Methanol and acetonitrile were HPLC grade and obtained through Fisher Scientific (Pittsburgh,PA 15219).

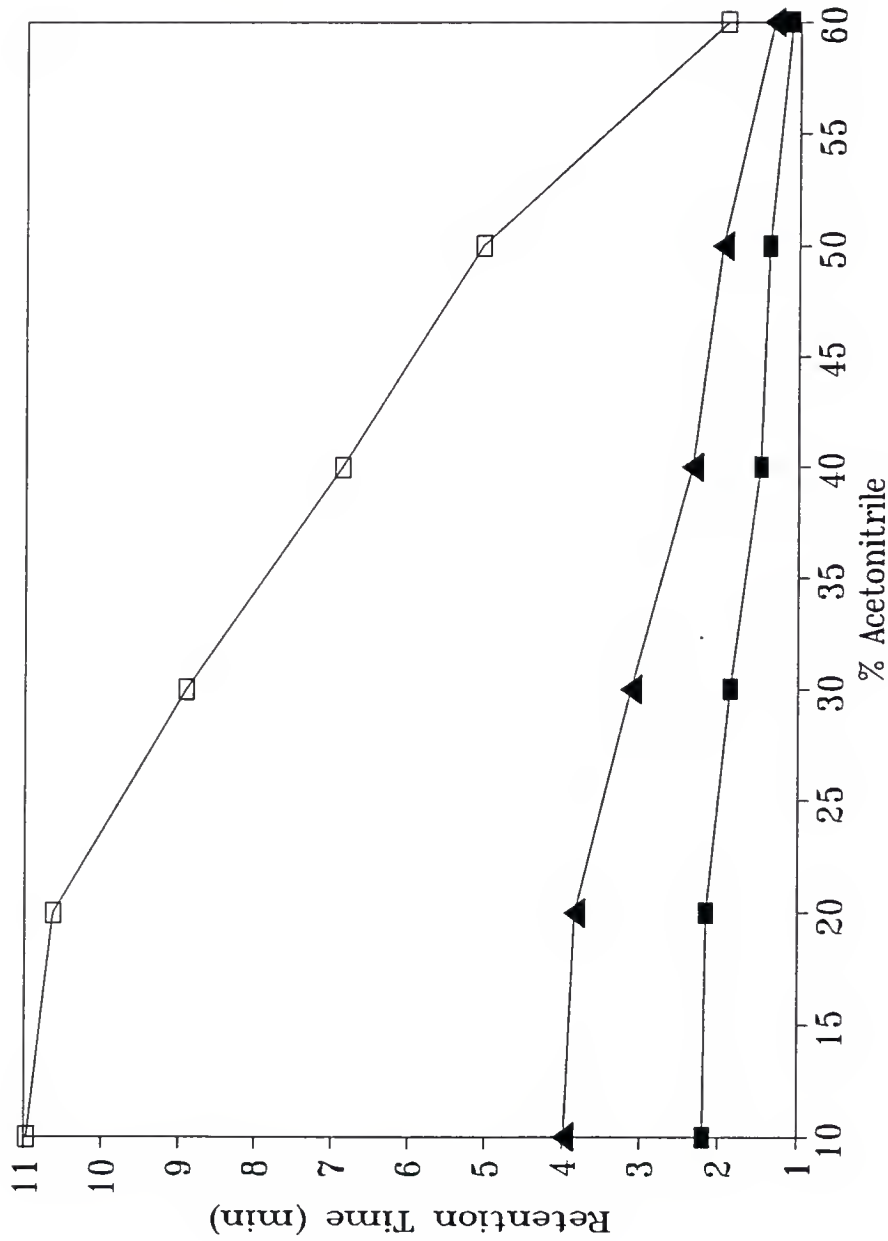
Optimization of Parameters

The probe species IR-125 which was used with the CZE system in Chapter 2 was also used with this IC system. To check the alignment of the flow cell and detector optics, injections of IR-125 in methanol were made into an eluent of 50 mM NaOH and

50 % methanol with no columns present. The detection limit for IR-125 with these conditions was in the femtomole range. IR-125 was then injected onto the guard column only, with an eluent of 50 mM NaOH at 2.0 ml/min, and no peak was observed. When the guard column was rinsed with methanol to remove the IR-125, still, no peak was observed. The guard column was then rinsed with acetonitrile and immediately a very large peak was recorded. It appeared that some amount of acetonitrile was needed to keep the dye from being retained on the column and a change of eluent conditions was necessary.

Organic modifiers. The fluorescence of IR-125 was shown to increase with addition of acetonitrile or methanol. These solvents have been used in IC to change the separation parameters, and were investigated with respect to this system. Since initial results showed that acetonitrile was necessary in the IFD eluent, the effect of this solvent on the retention of SeO_4^{-2}, SeO_3^{-2} and $HAsO_4^{-2}$ was determined with suppressed conductivity detection. The effect of this solvent on the retention of AsO_2^{-1} could not be determined, since it was not possible to detect it by suppressed conductivity detection. Figure 3-2 shows that by increasing the amount of acetonitrile present in the eluent, the retention times of the analytes can be reduced. Above 60 % acetonitrile, the three peaks are no longer resolved. Also, at the higher amounts of acetonitrile, the detection of AsO_2^{-1} would not be possible, since it would be "lost" in the solvent front. With this in mind, the characteristics of IR-125 in eluents containing less than 60 % acetonitrile were investigated. The effect of acetonitrile on the background fluorescence signal of IR-125 was then determined. An eluent containing 5.2×10^{-7} M IR-125 and 5 mM NaOH was

Figure 3-2: Effect of acetonitrile on the retention of arsenic and selenium oxyanions with a gradient of 10 mM NaOH at t=0 min to 20 mM NaOH at t=20 min: (■) SeO_3^{-2}; (▲) SeO_4^{-2}; and (□) $HAsO_4^{-2}$.


Retention Time (min)
11
10
9
8
7
6
5
4
3
2
1
10
15
20
25
30
35
40
45
50
55
60
% Acetonitrile

pumped through the column. The percentage of acetonitrile present in the eluent was increased over time. The results are shown graphically in Figure 3-3. It was then thought that a mixture of acetonitrile and methanol might enhance the background signal, while allowing good separation of the analytes. An eluent containing 5.2 x 10^{-7} M IR-125, 5 mM NaOH, and 10 % acetonitrile was pumped through the column. The percentage of methanol was increased, over time, and the fluorescence background signal was recorded. This was repeated with an eluent containing 20 % acetonitrile. The results are shown graphically in Figure 3-4. These figures show that with a higher percentage of organic modifier, the dye is not retained as strongly, and the fluorescence signal increases.

Separations. Detection of the arsenic and selenium oxyanions was attempted with various combinations of methanol, acetonitrile and sodium hydroxide. Unfortunately, separation and detection of all four anions was not achieved. With an eluent of 5.2 x 10^{-7} M IR-125, 5 mM NaOH, 50 % acetonitrile and 10% methanol, separation of SeO_3^{-2}, SeO_4^{-2}, and $HAsO_4^{-2}$ was performed, as shown in Figure 3-5. In another case, an eluent containing 5.2 x 10^{-7} M IR-125, 5 mM NaOH, 20 % acetonitrile and 30% methanol was used to separate and detect SeO_3^{-2} and SeO_4^{-2}, as shown in Figure 3-6. The noise which is observed is bit-noise, due to the electronics, and the magnification of the signal. The signals which were observed for the analytes were small, and most of the time, were actually a dip followed by a peak, instead of the expected dip. It appeared that there was only a very local displacement of the dye species, which would explain the peaks which followed the dips in the chromatogram. Ideally, the displaced dye should be seen at the

Figure 3-3: Effect of increasing percentage of acetonitrile on the background fluorescence signal of an eluent containing 5.2×10^{-7} M IR-125 and 5 mM NaOH.

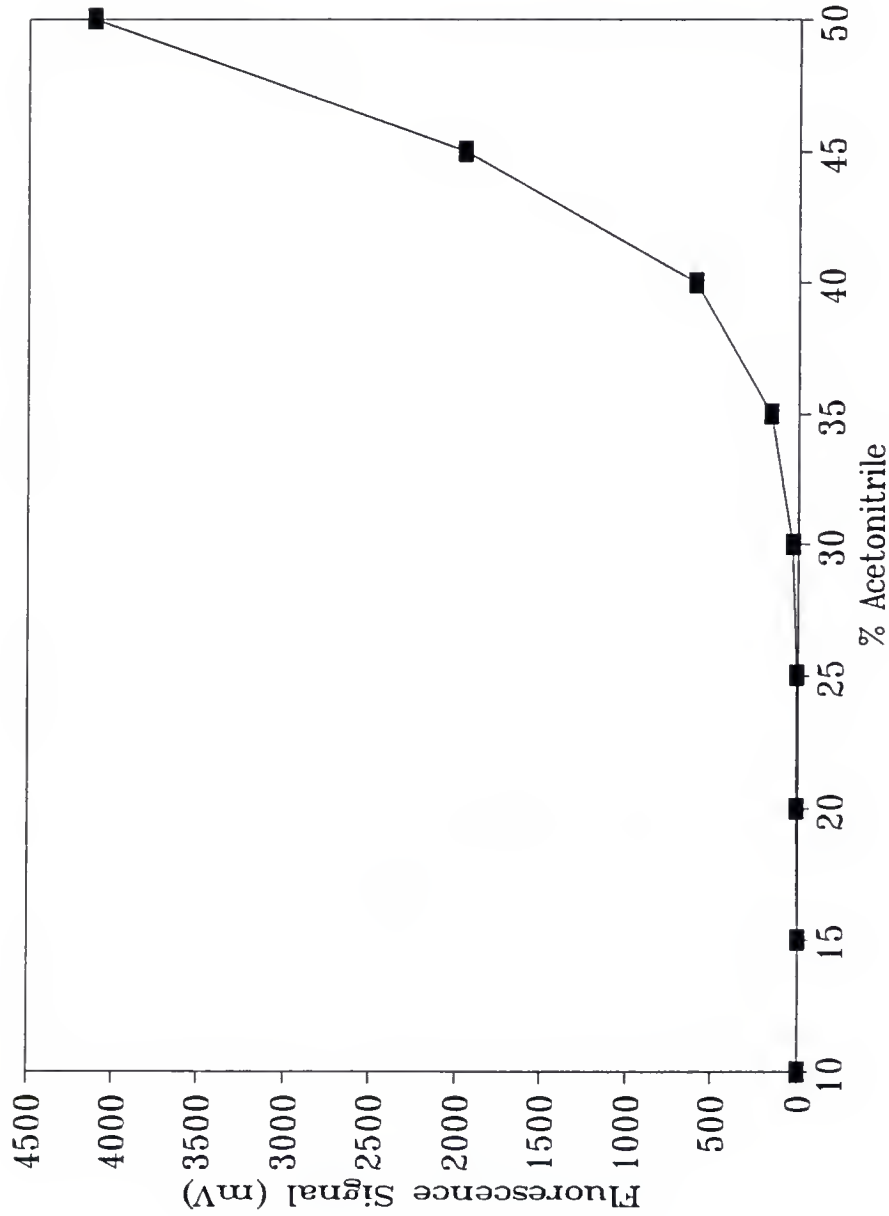

Fluorescence Signal (mV)
4500
4000
3500
3000
2500
2000
1500
1000
500
0
10
15
20
25
30
35
40
45
50
% Acetonitrile

Figure 3-4: Effect of increasing percentage of methanol on the fluorescence background signal of an eluent containing 5.2×10^{-7} M IR-125, 5 mM NaOH, and acetonitrile: (■) 10 % acetonitrile; (▲) 20 % acetonitrile.

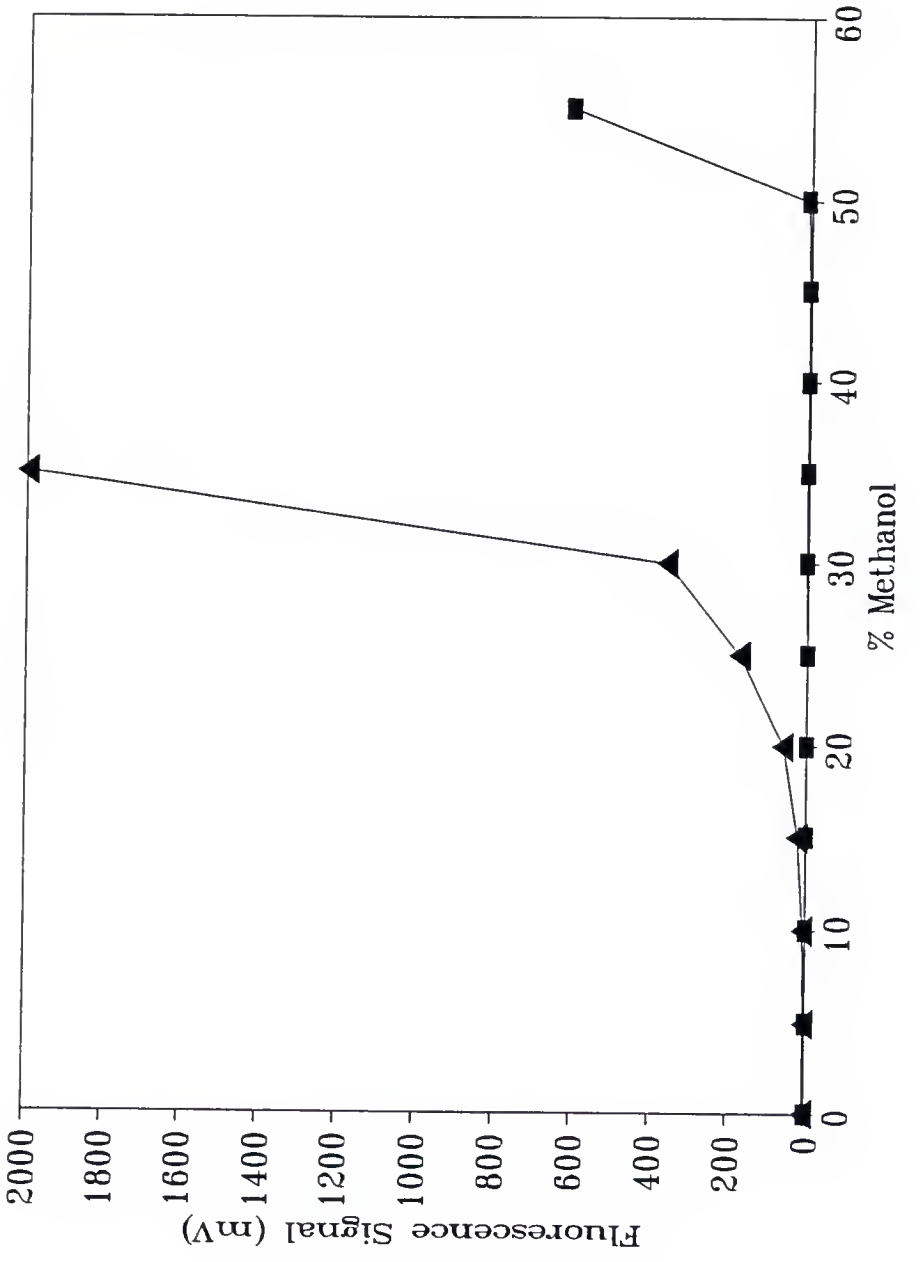
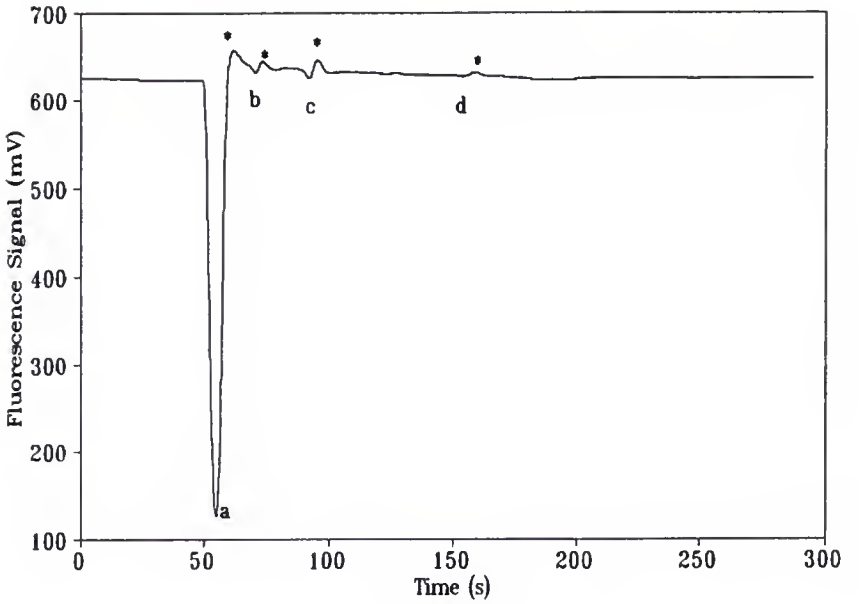

Fluorescence Signal (mV)
2000
1800
1600
1400
1200
1000
800
600
400
200
0
0
10
20
30
40
50
60
% Methanol

Figure 3-5: Chromatogram of 20 ppm arsenic/selenium oxyanion mixture with an eluent containing 5.2 x 10^{-7} M IR-125, 5 mM NaOH, 50 % acetonitrile, and 10 % methanol: (A) full scale view; (B) magnified view. Signals designated as: (a) solvent front; (b) SeO_3^{-2}; (c) SeO_4^{-2}; (d) $HAsO_4^{-2}$; (*) displaced IR-125.

A


Fluorescence Signal (mV)
700
600
500
400
300
200
100
0
50
100
150
200
250
300
Time (s)
a
b
c
d

B

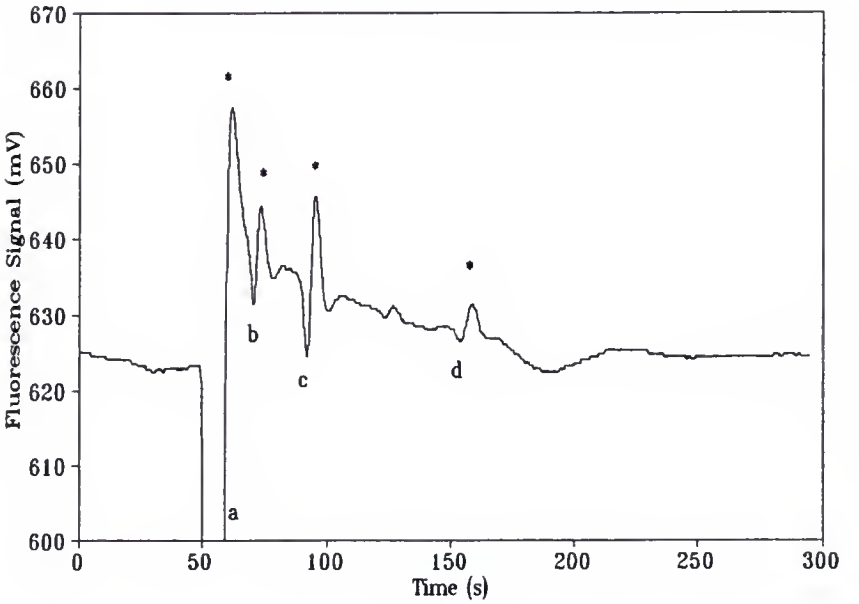
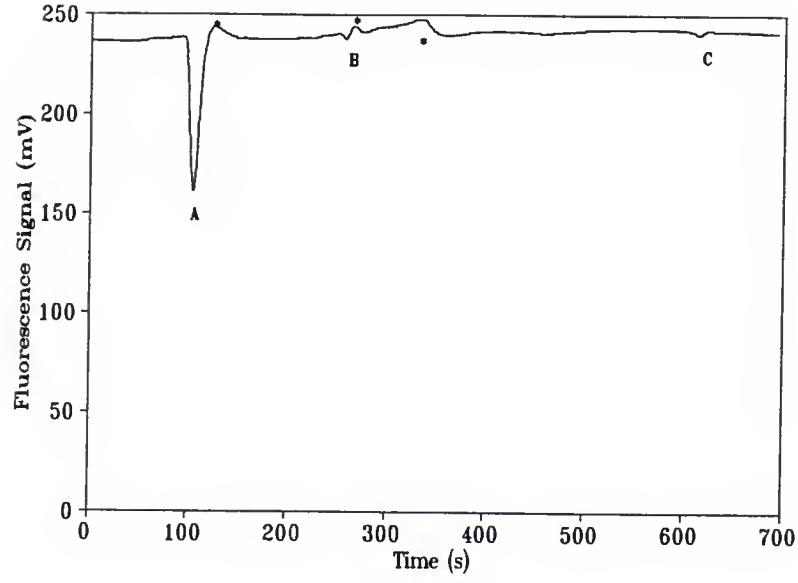
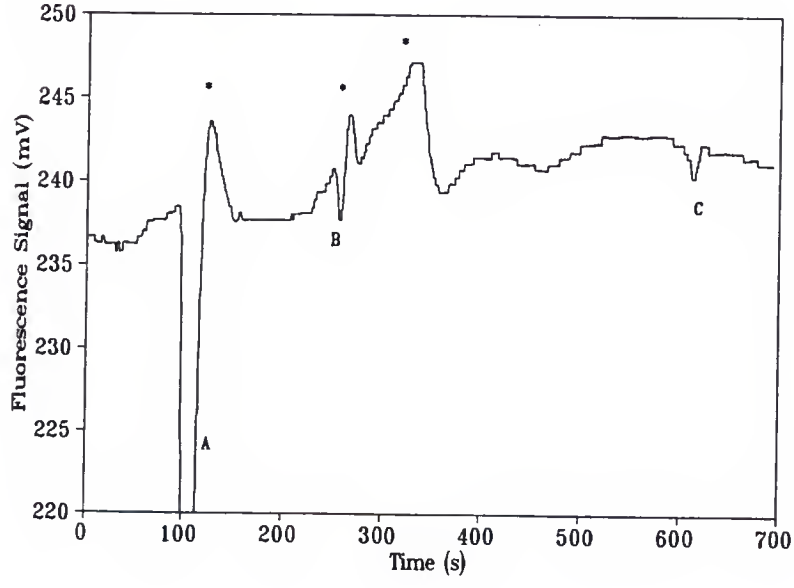

Fluorescence Signal (mV)
670
660
650
640
630
620
610
600
0
50
100
150
200
250
300
Time (s)
a
b
c
d

Figure 3-6: Chromatogram of an arsenic/selenium oxyanion mixture with eluent of 5.2×10^{-7} M IR-125, 5 mM NaOH, 20 % acetonitrile, and 35 % methanol: (A) full scale view; (B) magnified view. Signals designated as: (a) solvent front; (b) 13 ppm SeO_3^{-2}; (c) 14 ppm SeO_4^{-2}; (*) displaced IR-125.

A


Fluorescence Signal (mV)
250
200
150
100
50
0
A
B
C
0
100
200
300
400
500
600
700
Time (s)

B


Fluorescence Signal (mV)
250
245
240
235
230
225
220
A
B
C
0
100
200
300
400
500
600
700
Time (s)

retention time of the dye, but this was not the case in this system. Several eluent systems were investigated, but it soon became apparent that this probe would not be useful for the analysis of these arsenic and selenium oxyanions in conjunction with IC.

Discussion

The use of a diode laser for excitation has the potential for allowing lower detection limits to be achieved with indirect fluorometric detection due to its stability. With IC, however, there appears to be an incompatibility between the probes which can be used with these near-IR lasers, and the chromatography involved in the separation of small inorganic anions. The retention characteristics of these analytes and the IR-125 are too dissimilar to successfully use indirect detection for their determination. A smaller, more water soluble, near-IR dye might offer a means of indirect detection, or the availability of a diode laser at a shorter wavelength, so that probe species more similar to the arsenic and selenium oxyanions could also provide what is necessary to make this analysis work.

Using a He-Cd Laser as the Excitation Source

As with the CZE system, the use of a He-Cd laser as an excitation source for IFD of arsenic and selenium anions was evaluated. Since the He-Cd laser efficiently excites molecules at 325 nm, probes closer in retention characteristics to the analytes of interest were evaluated.

Experimental Section

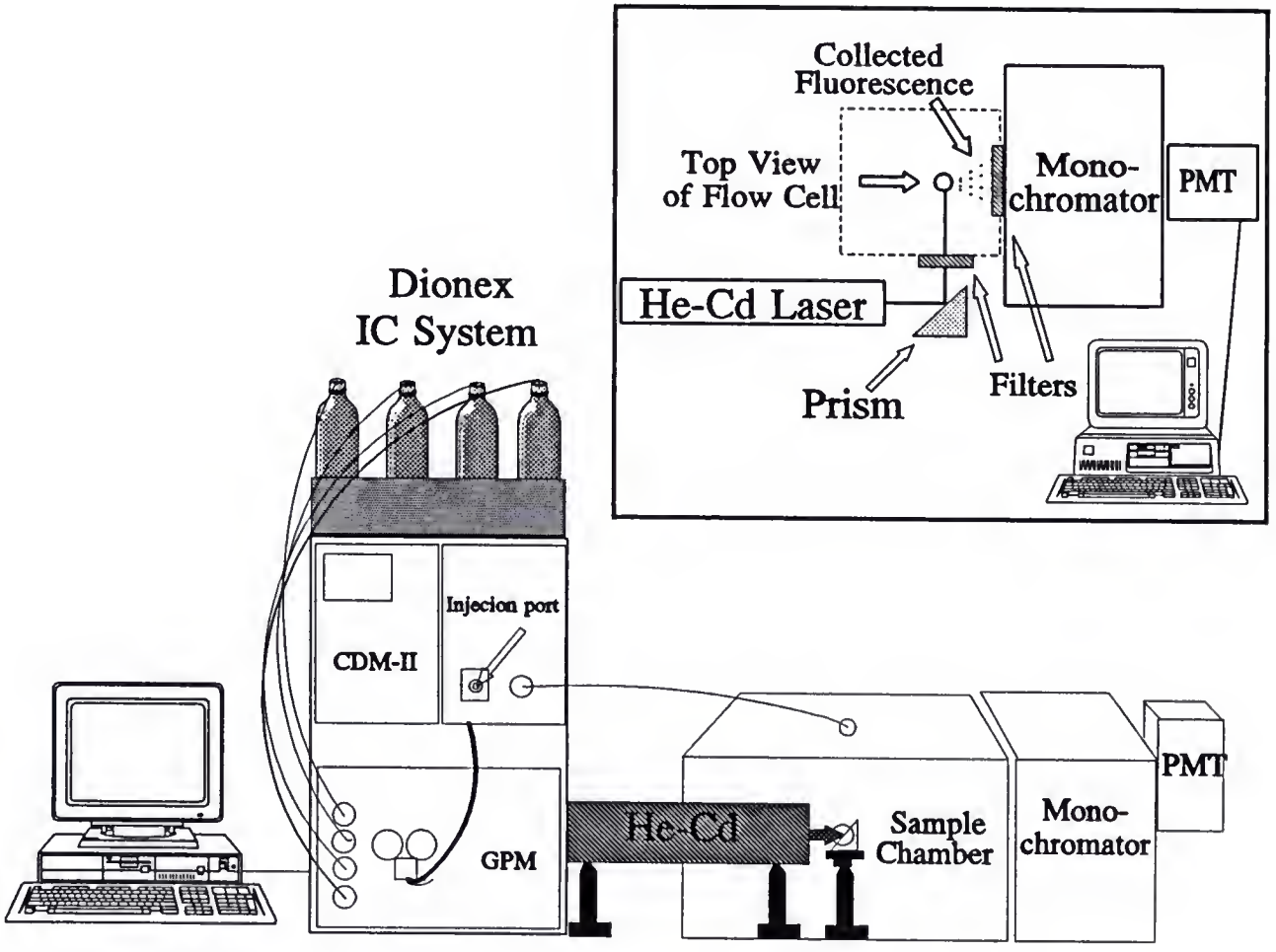
Chromatography system. The schematic for this system is shown in Figure 3-7. The same Dionex Corporation Ion Chromatography system (Sunnyvale,CA 94088) which was described earlier in this chapter was used for these experiments.

Excitation system. A Liconix He-Cd laser (Santa Clara,CA 94089) was used as the excitation source. This is the same source which was used with the CZE system in Chapter 2. Again, the 325 nm line was utilized. During the course of experimentation, the laser was returned to the manufacturer for repair. The power output was decreasing over time, and the internal optics had to be replaced and reoptimized.

Detection system. A flow cell was made from a section of Polymicro Technologies fused silica capillary (Phoenix,AZ 85017). The outer diameter of the capillary was 360 μm, and the inner diameter was 250 μm. This flow cell was placed in the sample chamber of the SPEX Fluorolog spectrophotometer (Edison,NJ 08820) with a specially designed holder. The SPEX system was the same as described in Chapter 2. The CDM-I Dionex conductivity detector was used in conjunction with the IFD for some experiments. Because of the high conductivity of the eluents used for this analysis, indirect conductometric detection was observed.

Chemicals. The analyte solutions were made from their sodium salts. Sodium arsenite, sodium arsenate, sodium selenite, sodium selenate, and sodium salicylate were obtained from Sigma Corporation (St.Louis,MO 63178). Sulfosalicylic acid was obtained from Mallincrodt (Chesterfield,MO 63017), and m-hydroxybenzoic acid was obtained from Kodak (Rochester,NY 14650). Sodium hydroxide was made from 50% solutions

Figure 3-7: Illustration of the He-Cd based system. Inset contains schematic of the instrumentation.


Collected
Fluorescence
Top View
of Flow Cell
Mono-
chromator
PMT
He-Cd Laser
Prism
Filters
Dionex
IC System
Injecion port
CDM-II
GPM
He-Cd
Sample
Chamber
Mono-
chromator
PMT

obtained from Fisher Scientific (Pittsburgh,PA 15219). The pH was adjusted, when necessary, with a dilute solution of sodium hydroxide. Barnstead nanopure water (Dubuque,Iowa 52004) was used to prepare all of the solutions.

Results

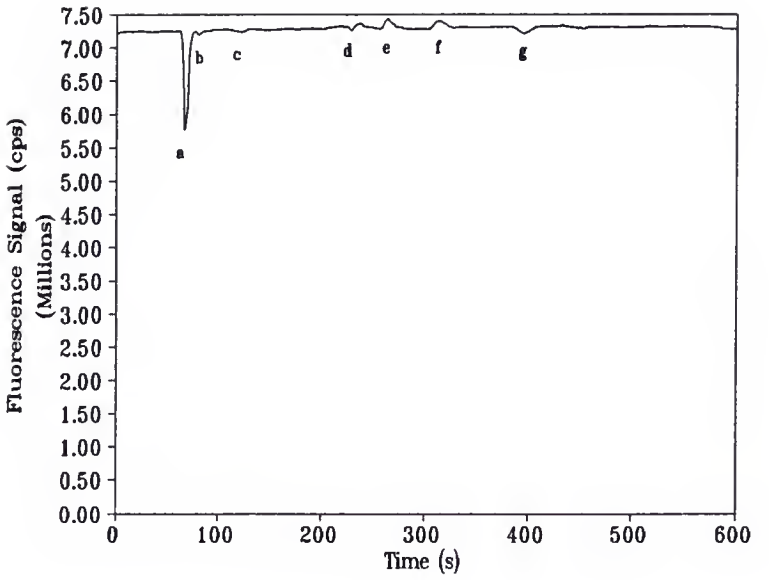
Three different probe species were evaluated with this system. They are the same as was used in the CZE/He-Cd laser based system described in Chapter 2. Each has retention characteristics which are more similar to the analytes than that of IR-125.

Salicylate. Salicylate was chosen since it has been used as an IC eluent in the literature. This allowed a comparison of our system with others. With an eluent consisting of 1.0 mM salicylate and 4 mM NaOH separation and detection of AsO_2^{-1}, SeO_3^{-2}, and SeO_4^{-2} was performed. The chromatogram is shown in Figure 3-8. While the salicylate probe allowed separation of these anions, the background fluorescence signal was not as sufficiently stable for analytical measurements.

Sulfosalicylate. Since it was possible to separate and detect these anions with the salicylate, the evaluation of several new probes was begun. With an eluent containing 1.0 mM sulfosalicylate at pH 9.4 the separation of SeO_3^{-2}, SeO_4^{-2}, and $HAsO_4^{-2}$ was achieved, as shown in Figure 3-9. The detection of AsO_2^{-1} was not possible due to the large solvent front. In order to reduce the solvent strength, and separate the AsO_2^{-1} from the solvent front, the concentration of sulfosalicylate was decreased. At a concentration of 0.50 mM sulfosalicylate, the AsO_2^{-1} was still not detectable. This separation is shown in Figure 3-10. With an eluent containing 0.25 mM sulfosalicylate at pH 9.4, the

Figure 3-8: Chromatogram of an arsenic/selenium oxyanion mixture with an eluent of 1.0 mM salicylate and 4.0 mM NaOH at 2 ml/min: (A) full scale view; (B) magnified view. Signals designated as: (a) solvent front; (b) 21 ppm AsO_2^{-1}; (c) NO_2^{-1} impurity; (d) 20 ppm SeO_3^{-2}; (e) unidentified; (f) salicylate; (g) 20 ppm SeO_4^{-2}.

A


Fluorescence Signal (cps)
(Millions)
7.50
7.00
6.50
6.00
5.50
5.00
4.50
4.00
3.50
3.00
2.50
2.00
1.50
1.00
0.50
0.00
a
b
c
d
e
f
g
0
100
200
300
400
500
600
Time (s)

B

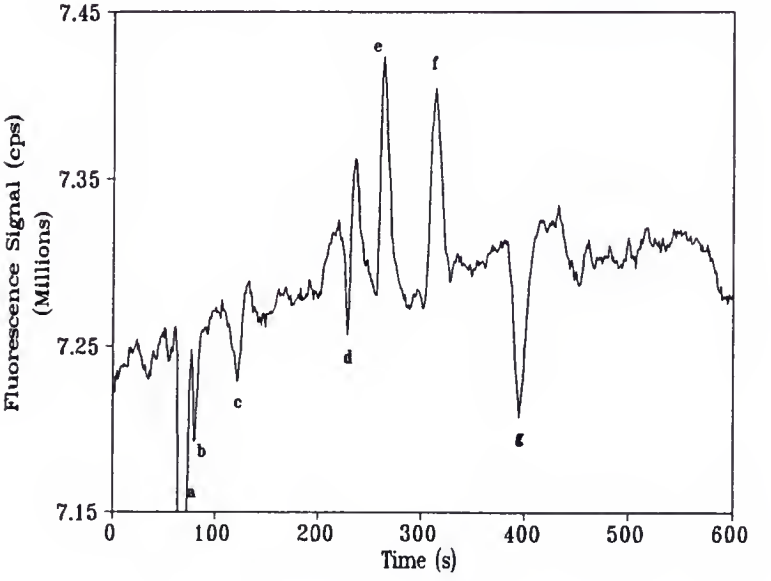
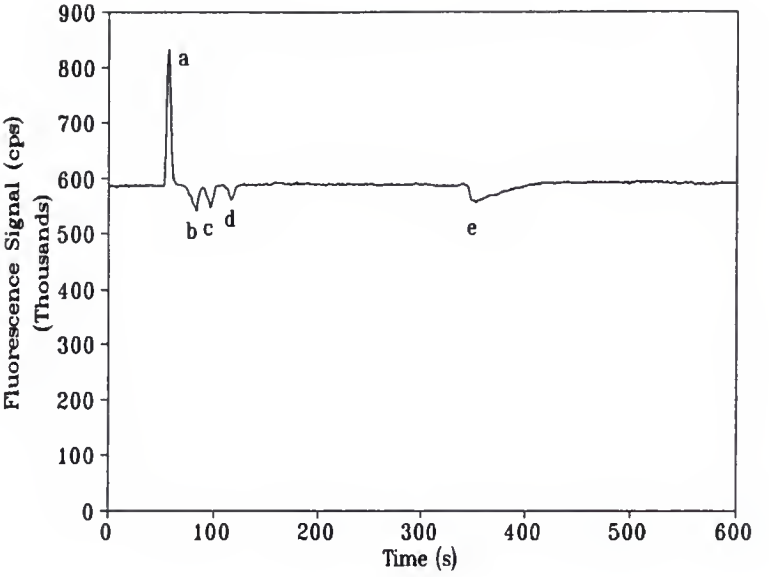

Fluorescence Signal (cps)
(Millions)
7.45
7.35
7.25
7.15
a
b
c
d
e
f
g
0
100
200
300
400
500
600
Time (s)

Figure 3-9: Chromatogram of a 60 ppm arsenic/selenium oxyanion mixture with an eluent of 1.0 mM sulfosalicylate at pH 9.4 at 2.5 mL/min: (A) full scale view; (B) magnified view. Signals designated as: (a) solvent front; (b) sulfosalicylate; (c) SeO_3^{-2}; (d) SeO_4^{-2}; (e) $HAsO_4^{-2}$.

A


900
800
700
600
500
400
300
200
100
0
Fluorescence Signal (cps)
(Thousands)
a
b c d
e
0
100
200
300
400
500
600
Time (s)

B

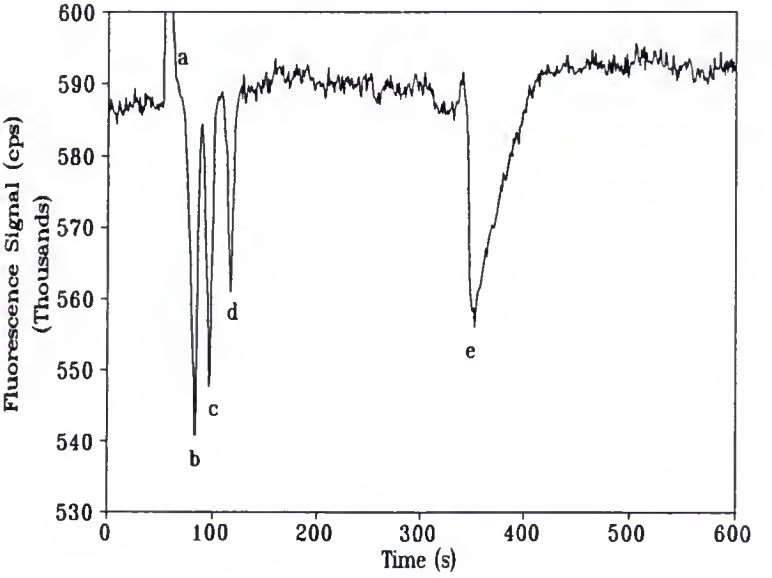
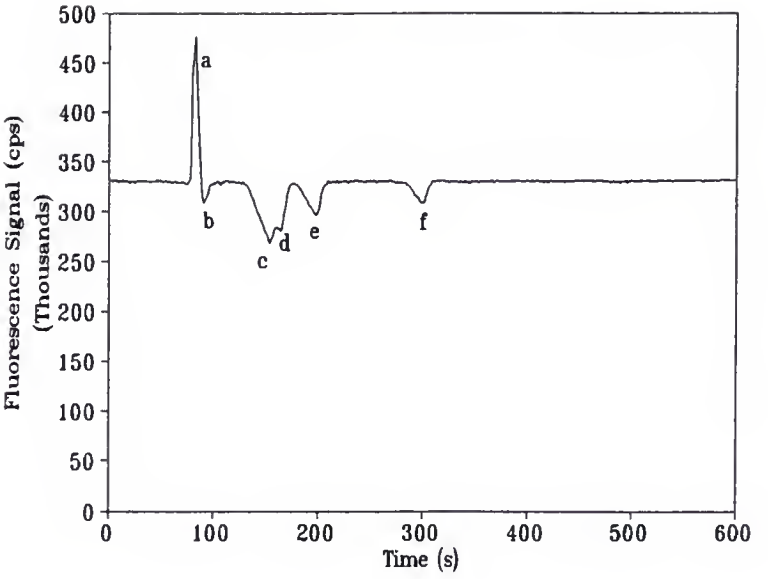
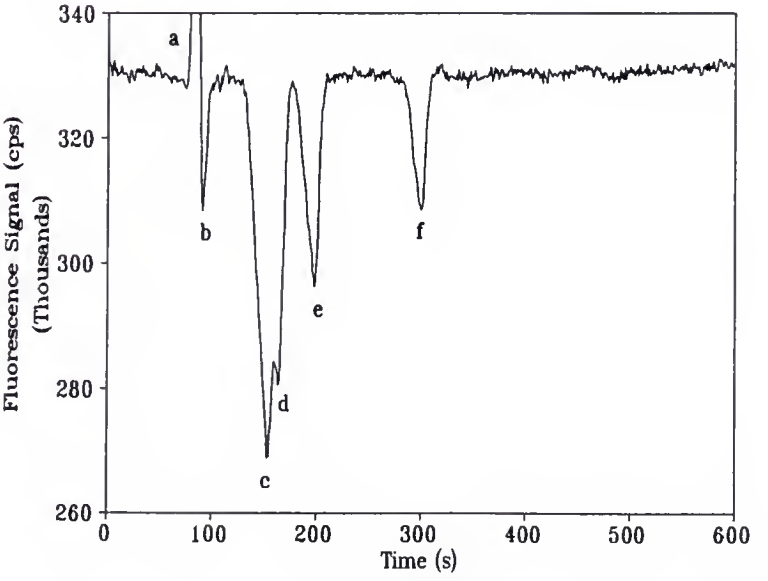

600
590
580
570
560
550
540
530
Fluorescence Signal (cps)
(Thousands)
a
b
c
d
e
0
100
200
300
400
500
600
Time (s)

Figure 3-10: Chromatogram of a 20 ppm arsenic/selenium oxyanion mixture with an eluent of 0.5 mM sulfosalicylate at pH 9.4 at 2.5 mL/min: (A) full scale view; (B) magnified view. Signals designated as: (a) solvent front; (b) AsO_2^{-1}; (c) sulfosalicylate; (d) SeO_3^{-2}; (e) SeO_4^{-2}; (f) $HAsO_4^{-2}$.

A


Fluorescence Signal (cps)
(Thousands)
500
450
400
350
300
250
200
150
100
50
0
a
b
c
d
e
f
0
100
200
300
400
500
600
Time (s)

B


Fluorescence Signal (cps)
(Thousands)
340
320
300
280
260
a
b
c
d
e
f
0
100
200
300
400
500
600
Time (s)

detection of all four analytes was achieved. This chromatogram is shown in Figure 3-11. Calibration curves were generated under these conditions. The experimental results are given in Table 3-1. The sulfosalicylate concentration in the eluent was then decreased to 0.10 mM, but this produced a chromatogram with a noisier background signal, and results that were worse than those obtained with the 0.25 mM sulfosalicylate.

Table 3-1: Results using eluent of 0.25 mM sulfosalicylate at pH 9.4 at 2.0 ml/min.

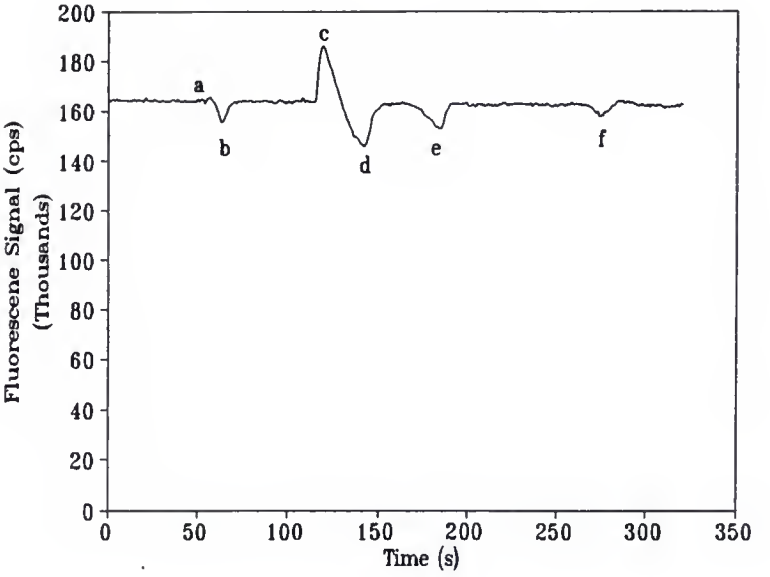
Analyte	k'	N	CLOD[a]	CLOD[b]	MLOD[c]
AsO_2^{-1}	0.12	500	42.	4.5	2.1
SeO_3^{-2}	1.60	700	3.0	0.38	0.15
SeO_4^{-2}	2.42	700	2.0	0.29	0.098
$HAsO_4^{-2}$	4.15	4000	42.	5.9	2.1

[a] concentration limit of detection in μmol/L.
[b] concentration limit of detection in μg/mL.
[c] absolute limit of detection in nmol.

m-hydroxybenzoate. The final probe which was evaluated was m-hydroxybenzoate. Similar experimental conditions where used with this probe, as with the two previous ones. Several different concentrations of m-hydroxybenzoate where evaluated. A probe concentration of 1.0 mM was evaluated first. A chromatogram showing typical results for the separation of the arsenic and selenium oxyanions using this probe concentration is shown in Figure 3-12. The next concentration evaluated was 0.50 mM m-hydroxybenzoate at pH 9.4. A chromatogram demonstrating the separation

Figure 3-11: Chromatogram of a 20 ppm arsenic/selenium oxyanion mixture with an eluent of 0.25 mM sulfosalicylate at pH 9.4 at 2.5 mL/min: (A) full scale view; (B) magnified view. Signals designated as: (a) solvent front; (b) AsO_2^{-1}; (c) sulfosalicylate; (d) SeO_3^{-2}; (e) SeO_4^{-2}; (f) $HAsO_4^{-2}$.

A


Fluorescene Signal (cps)
(Thousands)
Time (s)
a
b
c
d
e
f

B

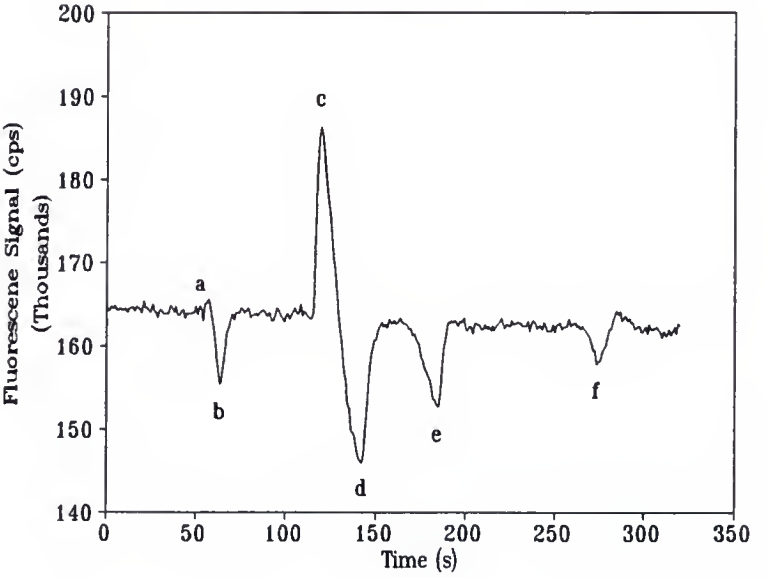
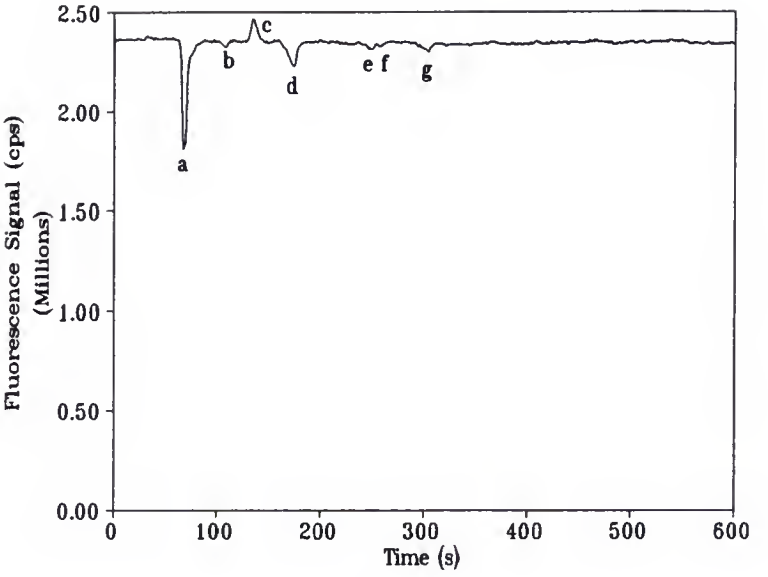
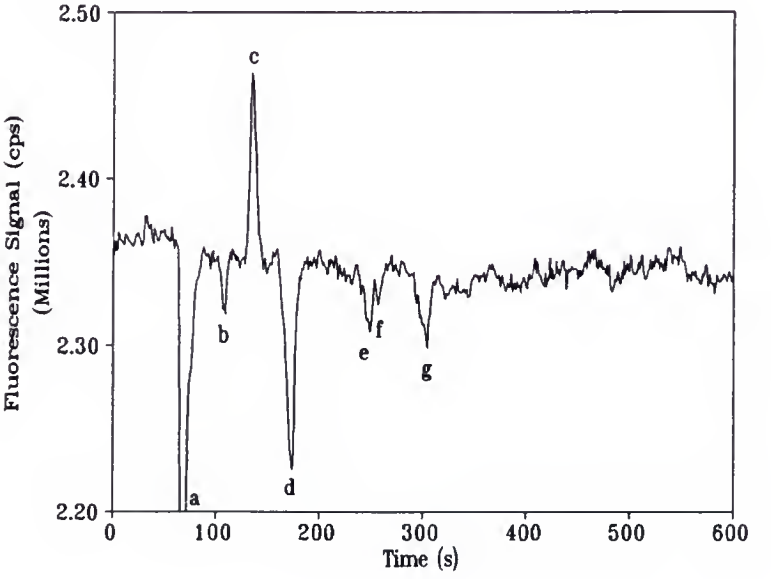

Fluorescene Signal (cps)
(Thousands)
Time (s)
a
b
c
d
e
f

Figure 3-12: Chromatogram of a 20 ppm arsenic/selenium oxyanion mixture with an eluent of 1.0 mM m-hydroxybenzoate at pH 9.4 at 2.0 mL/min: (A) full scale view; (B) magnified view. Signals designated as: (a) solvent front; (b) NO_3^{-1} (impurity); (c) m-hydroxybenzoate; (d) SeO_3^{-2}; (e) SeO_4^{-2}; (f) $H_2AsO_4^{-1}$; (g) $HAsO_4^{-2}$.

A


2.50
2.00
1.50
1.00
0.50
0.00
Fluorescence Signal (cps)
(Millions)
a
b
c
d
e f
g
0
100
200
300
400
500
600
Time (s)

B


2.50
2.40
2.30
2.20
Fluorescence Signal (cps)
(Millions)
a
b
c
d
e
f
g
0
100
200
300
400
500
600
Time (s)

of the arsenic and selenium oxyanions is shown in Figure 3-13. The best separations were achieved with this probe concentration. The probe concentration was decreased to 0.1 mM, but the separation and detection of these arsenic and selenium oxyanions was degraded, as shown in Figure 3-14.

Analytical calibration curves were constructed for these four anions, as well as for m-hydroxybenzoate, and are shown in Figures 3-15 to 3-19. These results were obtained using an eluent containing 0.5 mM m-hydroxybenzoate at pH 9.4. The experimental results are given in Table 3-2. With detection limits in the micromolar range, this system compares favorably with others used for this type of analysis.

Table 3-2: Results using eluent of 0.50 mM m-hydroxybenzoate at pH 9.4 at 2.0 ml/min.

Analyte	k'	N	CLOD[a]	CLOD[b]	MLOD[c]
AsO_2^{-1}	0.16	920	4.4	0.47	220
SeO_3^{-2}	3.22	2000	1.3	0.17	63
SeO_4^{-2}	5.70	2800	1.1	0.16	58
$HAsO_4^{-2}$	9.10	3400	2.5	0.35	130

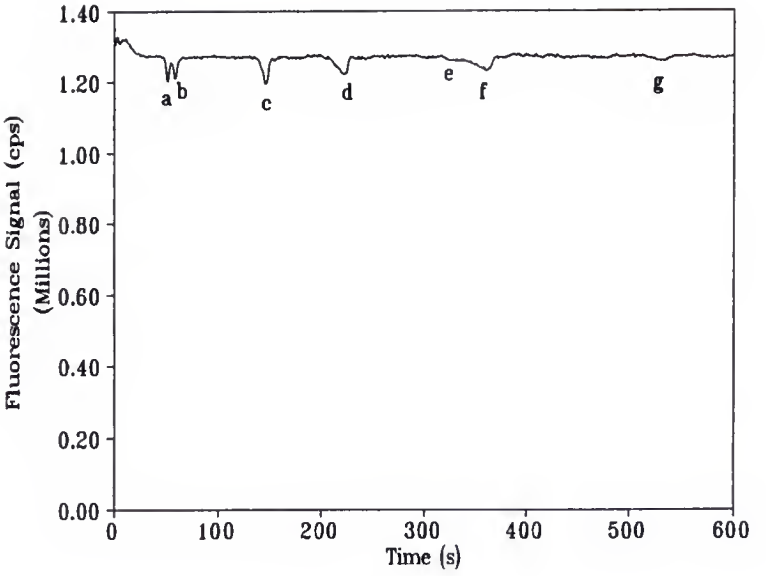
[a] concentration limit of detection in μmol/L.
[b] concentration limit of detection in μg/mL.
[c] absolute limit of detection in pmol.

Discussion

These experimental results have shown that the analysis of arsenic and selenium oxyanions can be accomplished by the use of IC with indirect fluorometric detection.

Figure 3-13: Chromatogram of a 20 ppm arsenic/selenium oxyanion mixture with an eluent of 0.5 mM m-hydroxybenzoate at pH 9.4 at 2.5 mL/min: (A) full scale view; (B) magnified view. Signals designated as: (a) solvent front; (b) AsO_2^{-1}; (c) m-hydroxybenzoate; (d) SeO_3^{-2}; (e) $H_2AsO_4^{-1}$; (f) SeO_4^{-2}; (g) $HAsO_4^{-2}$.

A


1.40
1.20
1.00
0.80
0.60
0.40
0.20
0.00
Fluorescence Signal (cps)
(Millions)
a
b
c
d
e
f
g
0
100
200
300
400
500
600
Time (s)

B

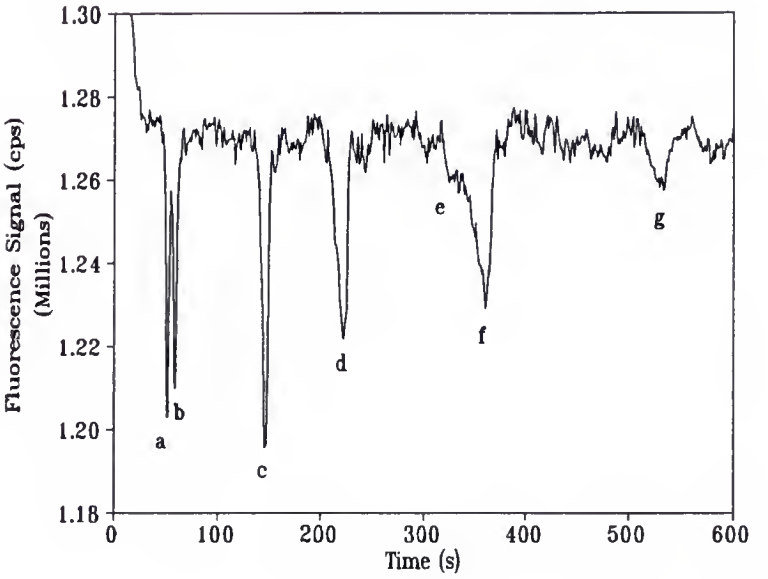
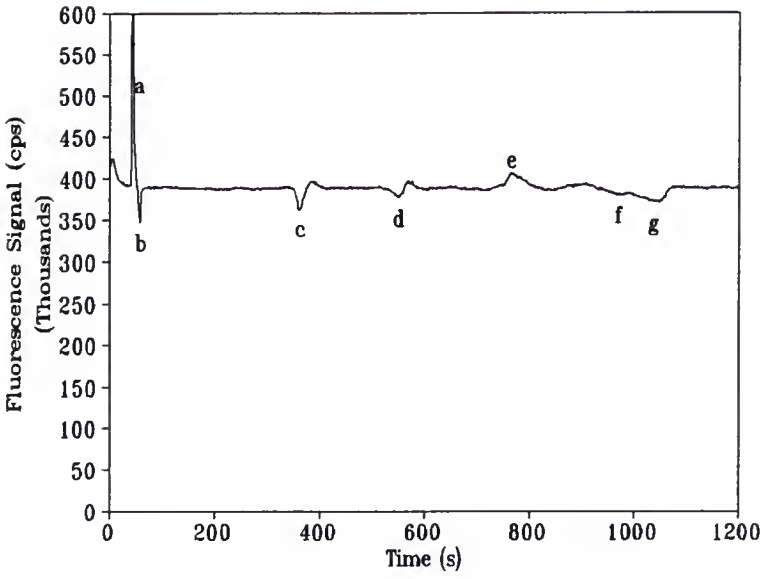

1.30
1.28
1.26
1.24
1.22
1.20
1.18
Fluorescence Signal (cps)
(Millions)
a
b
c
d
e
f
g
0
100
200
300
400
500
600
Time (s)

Figure 3-14: Chromatogram of a 20 ppm arsenic/selenium oxyanion mixture with an eluent of 0.1 mM m-hydroxybenzoate at pH 9.4 at 2.5 mL/min: (A) full scale view; (B) magnified view. Signals designated as: (a) solvent front; (b) AsO_2^{-1}; (c) SeO_3^{-2}; (d) $H_2AsO_4^{-1}$; (e) m-hydroxybenzoate; (f) SeO_4^{-2}; (g) $HAsO_4^{-2}$.

A


Fluorescence Signal (cps)
(Thousands)
600
550
500
450
400
350
300
250
200
150
100
50
0
0
200
400
600
800
1000
1200
Time (s)
a
b
c
d
e
f
g

B

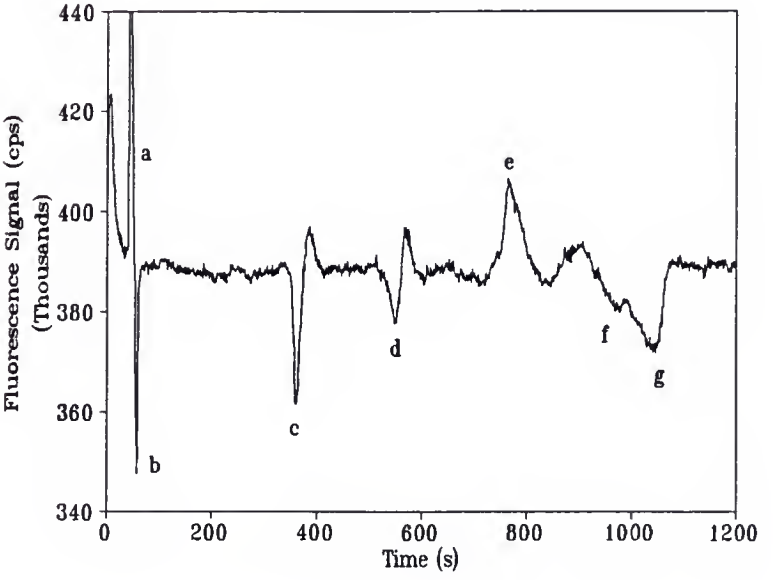

Fluorescence Signal (cps)
(Thousands)
440
420
400
380
360
340
0
200
400
600
800
1000
1200
Time (s)
a
b
c
d
e
f
g

Figure 3-15: Analytical calibration curve for AsO_2^{-1} with eluent of 0.50 mM m-hydroxybenzoate at pH 9.4 at 2.0 ml/min.
($Y = 2.18 \times 10^{13} X - 7.00 \times 10^{3}$; $R = 0.9903$)

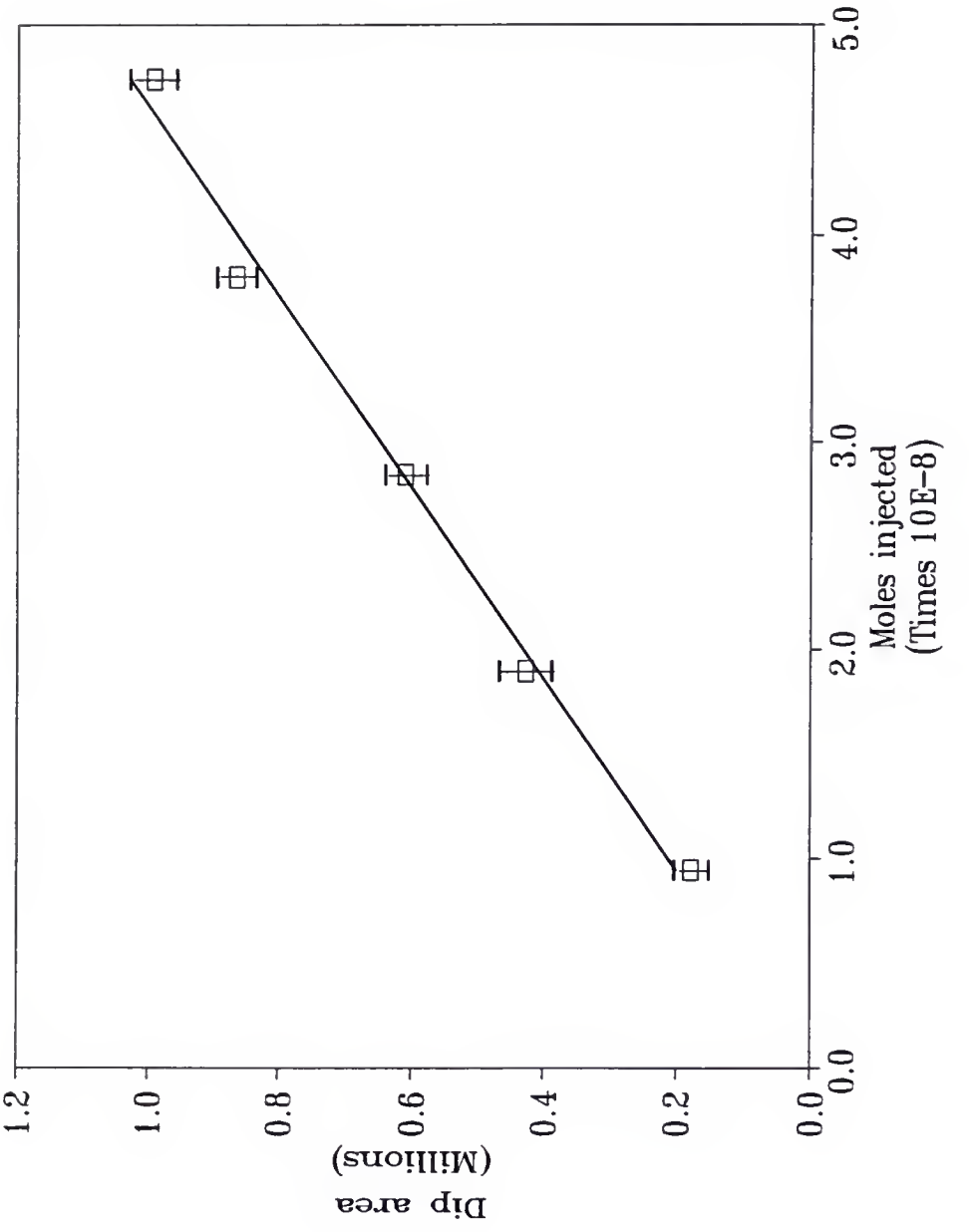

Dip area
(Millions)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0.0
1.0
2.0
3.0
4.0
5.0
Moles injected
(Times 10E-8)

Figure 3-16: Analytical calibration curve for SeO_3^{-2} with eluent of 0.50 mM m-hydroxybenzoate at pH 9.4 at 2.0 ml/min.
($Y = 7.68 \times 10^{13} X - 5.75 \times 10^{4}$; $R = 0.9937$)

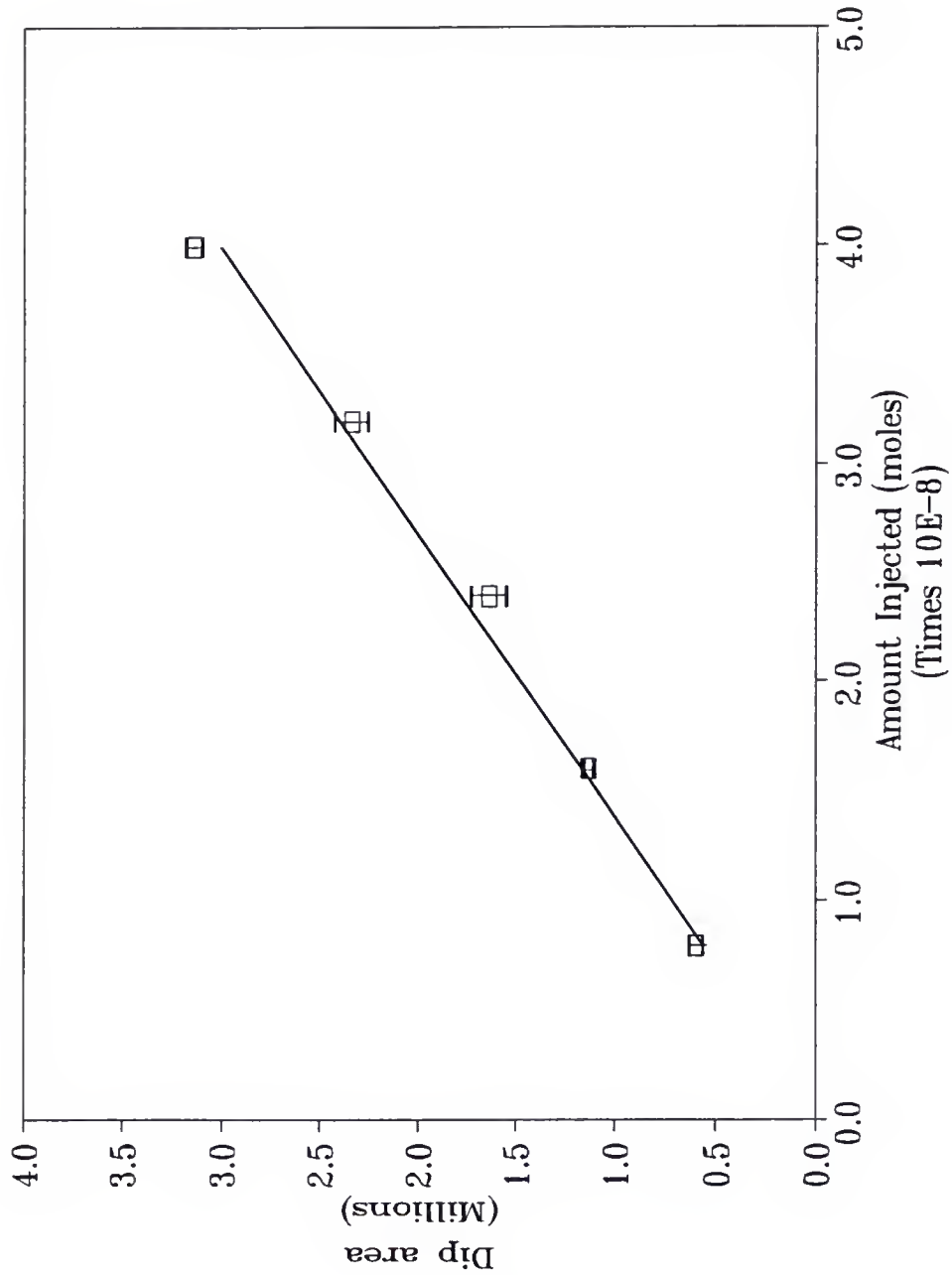

Dip area
(Millions)
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0.0
1.0
2.0
3.0
4.0
5.0
Amount Injected (moles)
(Times 10E-8)

Figure 3-17: Analytical calibration curve for SeO_4^{-2} with eluent of 0.50 mM m-hydroxybenzoate at pH 9.4 at 2.0 ml/min.
($Y = 8.54 \times 10^{13} X - 1.36 \times 10^{5}$; $R = 0.9920$)

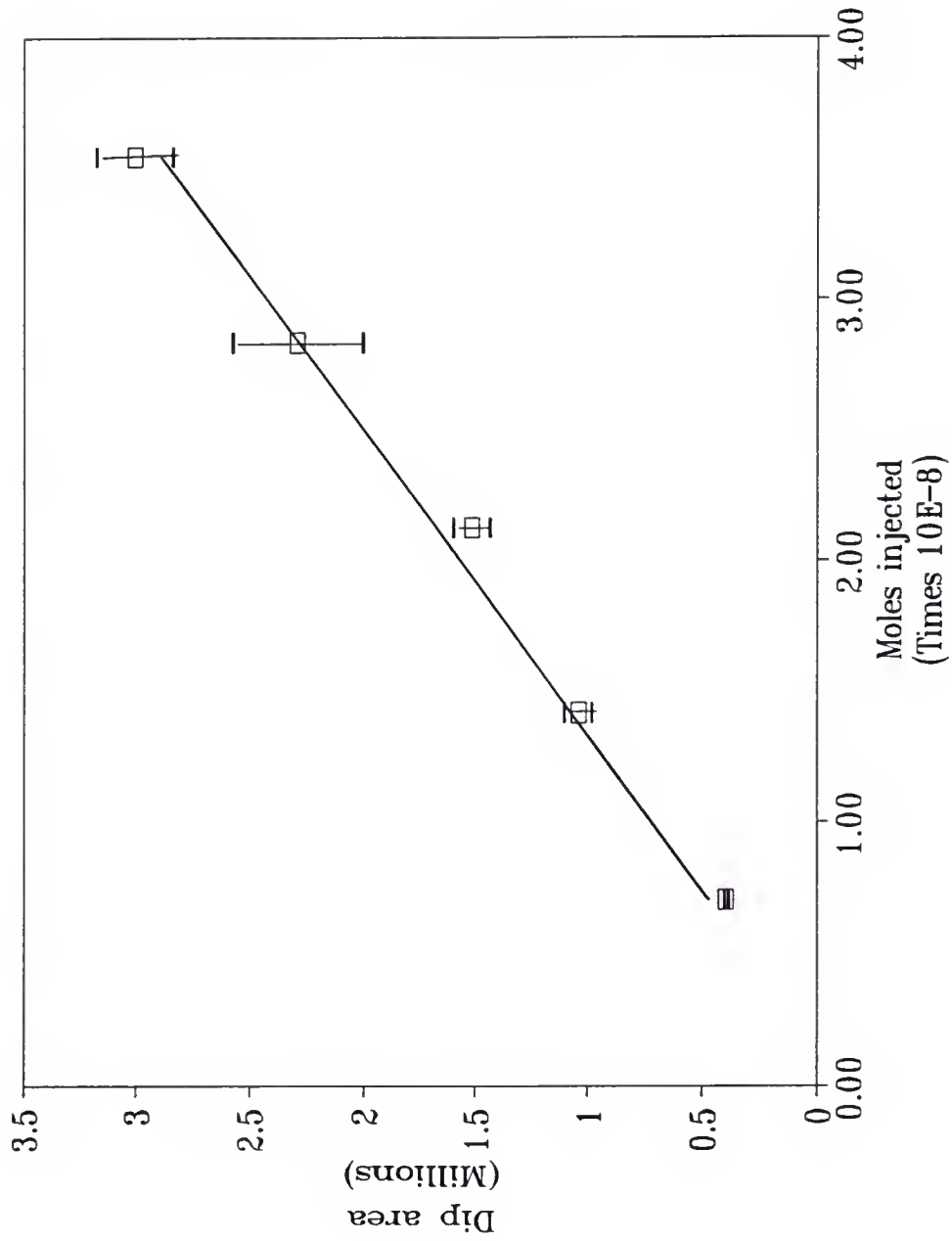

Dip area
(Millions)
3.5
3
2.5
2
1.5
1
0.5
0
0.00
1.00
2.00
3.00
4.00
Moles injected
(Times 10E-8)

Figure 3-18: Analytical calibration curve for $HAsO_4^{-2}$ with eluent of 0.50 mM m-hydroxybenzoate at pH 9.4 at 2.0 ml/min.
($Y = 3.84 \times 10^{13} X - 3.16 \times 10^{4}$; $R = 0.9747$)

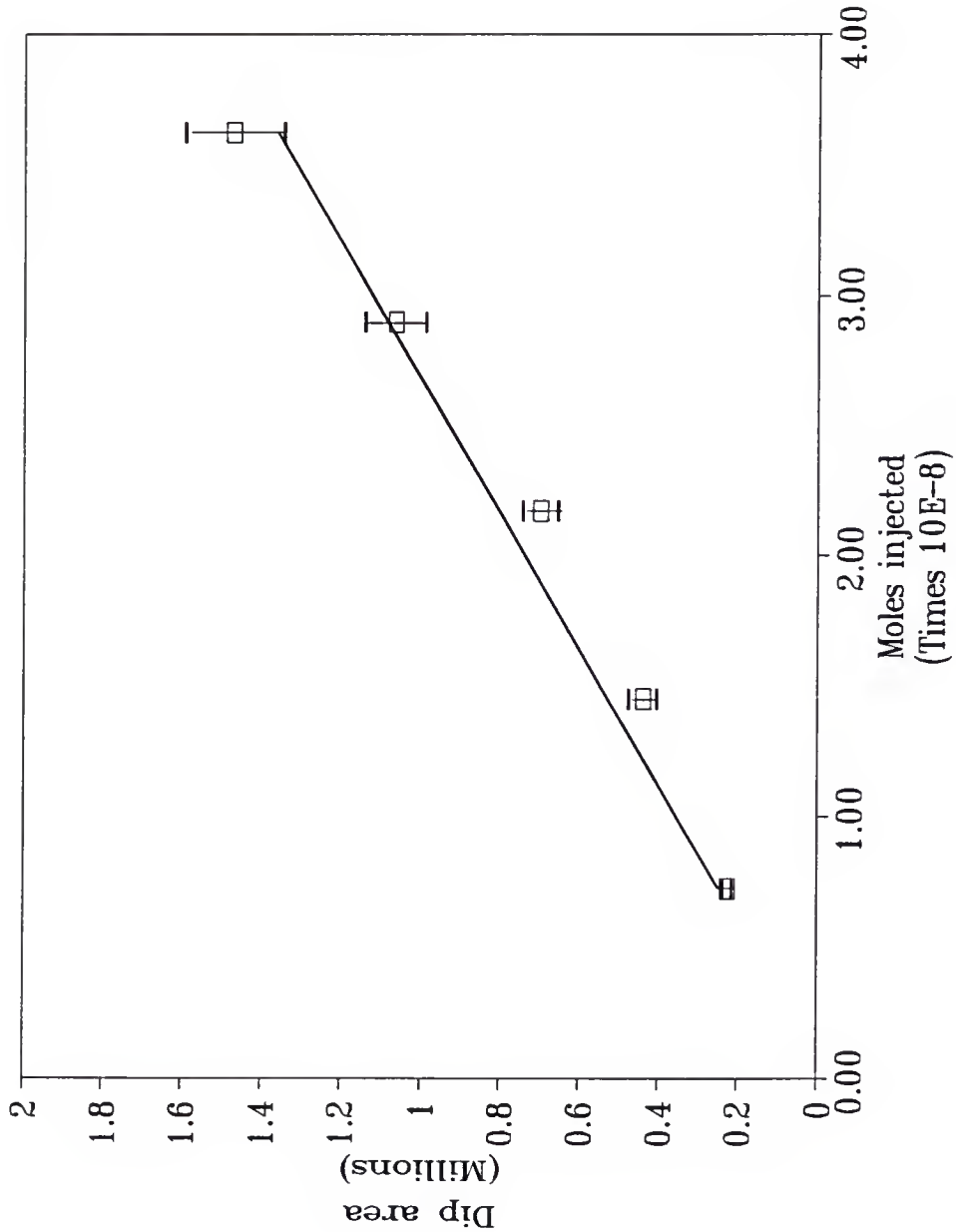
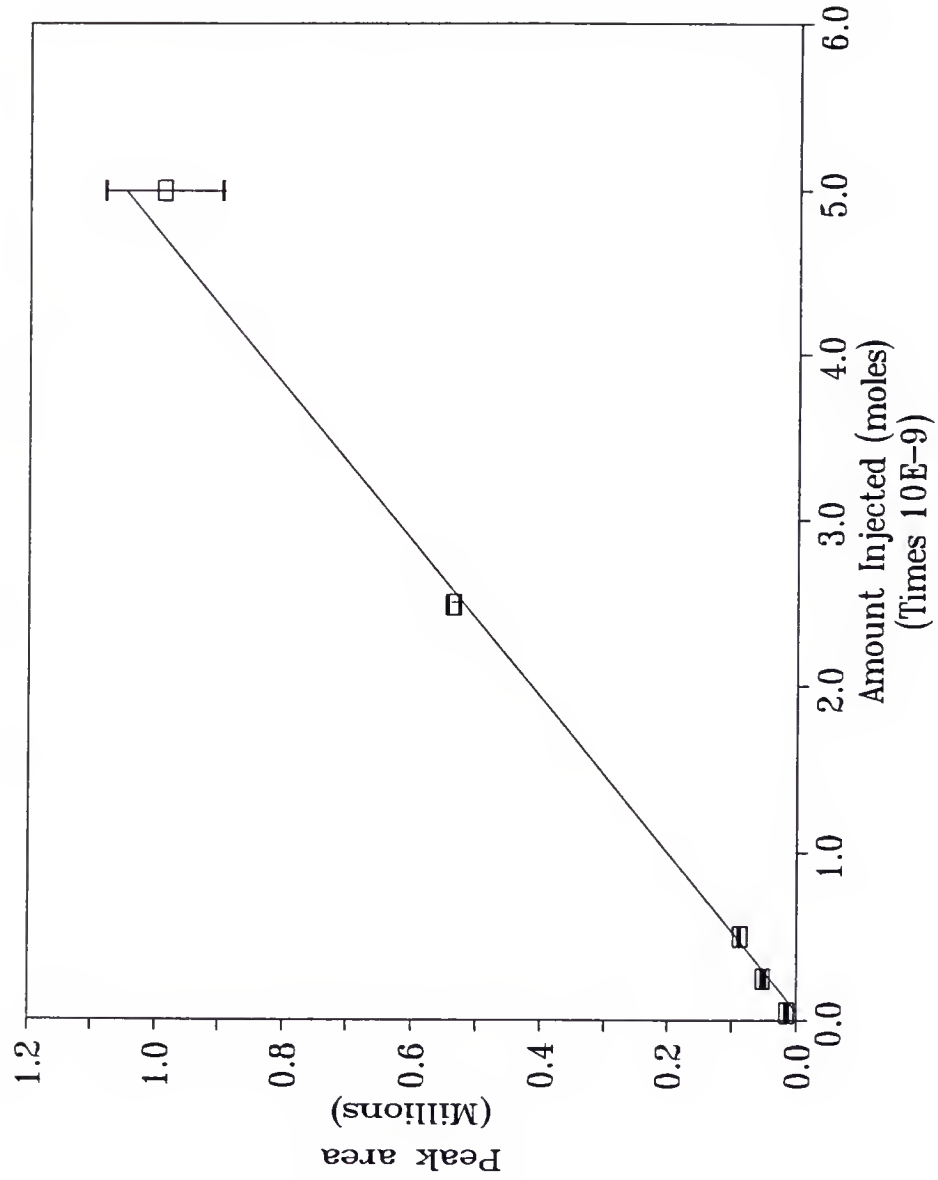

Dip area
(Millions)
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0.00
1.00
2.00
3.00
4.00
Moles injected
(Times 10E-8)

Figure 3-19: Analytical calibration curve for m-hydroxybenzoate. ($Y = 2.13 \times 10^{14} X - 1.29 \times 10^{4}$; $R = 0.9999$)


Peak area
(Millions)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0.0
1.0
2.0
3.0
4.0
5.0
6.0
Amount Injected (moles)
(Times 10E-9)

These measurements were performed with two different probes, sulfosalicylate and m-hydroxybenzoate, with the latter giving better detection limits. These detection limits, in the sub-ppm range, are comparable to those obtained by UV-vis absorption detection. As with the CZE experiments described in Chapter 2, the SPEX collection optics could be one of the problems which prevented lower detection limits to be achieved. A larger capillary was used a the flow cell in these experiments, than in with the CZE, which made placement in the sample chamber a bit easier. The signal due to the "blank", ie the signal observed when no fluorescent probe was present, was kept low with proper placement of the flow cell. The He-Cd laser noise was also a reason for the limited sensitivity. Addition of a laser power stabilizer could enhance the detection limits by increasing the DR of the system.

CHAPTER 4
CONCLUSIONS AND FUTURE WORK

The purpose of this dissertation was to investigate the use of indirect fluorometric detection for the detection of arsenic and selenium oxyanions in water samples. Through the use of the separation techniques of CZE and IC, the detection of arsenite, arsenate, selenite and selenate was successfully performed. It was hoped that through the use of a diode laser for excitation, the detection limits achieved for these four analytes could be improved. Unfortunately, the near-IR dyes which could be used with this type of excitation system had adverse reactions with the surface modifiers in the CZE system, and the stationary phase in the IC system. These adverse reactions prevented the separation and detection of the arsenic and selenium oxyanions with this diode laser-based system.

While this system was not successful for the analysis of inorganic anions, I believe that it still holds potential for the analysis of larger organic anions first separated by CZE. The larger organic ions would match closer, in both size and mobility, to the IR-125. This would eliminate the need to reverse the electroosmotic flow by the addition of a surface modifier (surfactant). It was the possible ion-pairing of the IR-125 with the surfactant which appeared to be the cause the most instability and irreproduciblity.

The research toward diode lasers which emit at shorter wavelengths continues. As diode lasers become available at shorter wavelengths different probes should be

evaluated with both CZE and IC separations with indirect fluorometric detection. The many advantages of diode lasers should prove beneficial for lowering the detection limits of this type of detector, if adverse reactions of the probe with the separating media are eliminated.

The use of a He-Cd laser-based system for excitation proved to be a better alternative, at this time, for the measurement of these arsenic and selenium oxyanions. A variety of probe species were evaluated with both types of separation systems (CZE and IC). One probe, m-hydroxybenzoate, was found to be the best probe to use, for both the IC and CZE separations. It provided the best separations, and the lowest detection limits (in the sub-ppm range). This research has succeeded in that the separation and detection of arsenite, arsenate, selenite, and selenate was achieved through the use of CZE and IC with indirect fluorometric detection using this probe.

Theory predicts that indirect fluorometric detection should be more sensitive that indirect absorption detection. This research does not show that, but, there is room for improvement in several areas of the system which should provide the lower detection limits in the future, as predicted by the theory.

The first improvement would be the addition of an output power stabilizer for the He-Cd laser. Better power stability would decrease the laser noise, and thereby increase the DR of the system. An increase in laser power would also decrease the detection limits. The higher power would increase the signal of the fluorescent background, which would also increase the DR.

A second improvement would be to further optimize the collection optics. It may be best to forgo the use of the SPEX system for the fluorescence collection, and setup more efficient collection optics for dealing specifically with a capillary as the flow cell. The optics in the SPEX system is designed for collection from a cuvette, not a capillary. Due to the small capillary diameters, especially in the CZE system, any movement of the capillary caused a change in the signal. A holder which secures the capillary better would be beneficial. The use of a microscope objective would be a better way of collecting the fluorescence signal. This type of arrangement would make optimization of the capillary placement easier than with the SPEX system. It is also likely to increase the solid angle of collection. The fluorescence could then be directed through a monochromator to a PMT, as in the SPEX system. A simpler option would be to use a series of interference filters before the PMT, instead of the monochromator, to select the range of wavelengths to be used for detection. This option would also increase the background signal, but more interferences would also be possible.

To analyze successfully water samples for arsenic and selenium oxyanions, detection limits in the sub-ppb range are necessary. The present system provides detection limits an order of magnitude too high. The aforementioned improvements could lower the detection limits to the necessary range. Once the detection limits are lowered, analysis of real samples could be performed. The determination of possible interferences when analyzing real samples could then be investigated.

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BIOGRAPHICAL SKETCH

MaryAnn Gunshefski (nee Richvalsky) was born on March 26, 1966, in Jersey City, NJ. She was raised in Bayonne, NJ, and graduated from Bayonne High School in 1984. She was awarded an academic scholarship from St. Peter's College, where she went on to earn a BS degree in chemistry, with a minor in mathematics, in May 1988. From August 1988 to June 1993 she attended graduate school at the University of Florida, where she studied analytical chemistry in the research group of Dr. James D. Winefordner. In August 1989, after her first year of graduate school, she married her high school sweetheart, Robert M. Gunshefski. In August of 1993 she received her Ph.D. in chemistry, with a minor in environmental engineering sciences. She accepted a position as Research Chemist at the National Council for Air and Stream Improvement in Gainesville, FL.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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Graduate Research Professor of Chemistry

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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